PCT WELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales Büro

INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation 6 : C07D 417/06, 493/04, C12P 17/08, A01N 43/78, A61K 31/425 // (C07D 493/04, 313:00, 303:00)

A1

(11) Internationale Veröffentlichungsnummer: **WO 98/22461**

(43) Internationales Veröffentlichungsdatum: 28. Mai 1998 (28.05.98)

(21) Internationales Aktenzeichen: PCT/EP97/06442

(22) Internationales Anmeldedatum: 18. November 1997 (18.11.97)

(30) Prioritätsdaten:

196 47 580.5	18. November 1996 (18.11.96)	DE
197 07 506.1	25. Februar 1997 (25.02.97)	DE

(71) Anmelder *(für alle Bestimmungsstaaten ausser US)*: GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE).

(72) Erfinder; und

(75) Erfinder/Anmelder *(nur für US)*: REICHENBACH, Hans [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE). HÖFLE, Gerhard [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE). GERTH, Klaus [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE). STEINMETZ, Heinrich [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE).

(74) Anwälte: BOETERS, Hans, D. usw.; Boeters & Bauer, Bereiteranger 15, D–81541 München (DE).

(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO Patent (GH, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Veröffentlicht

Mit internationalem Recherchenbericht.

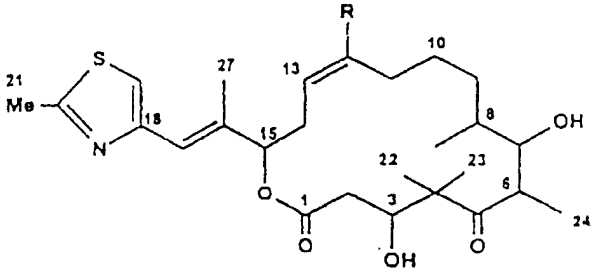
Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.

(54) Title: EPOTHILONE C, D, E AND F, PRODUCTION PROCESS, AND THEIR USE AS CYTOSTATIC AS WELL AS PHYTOSANITARY AGENTS

(54) Bezeichnung: EPOTHILONE C, D, E UND F, DEREN HERSTELLUNG UND DEREN VERWENDUNG ALS CYTOSTATISCHE MITTEL BZW. ALS PFLANZENSCHUTZMITTEL

(57) Abstract

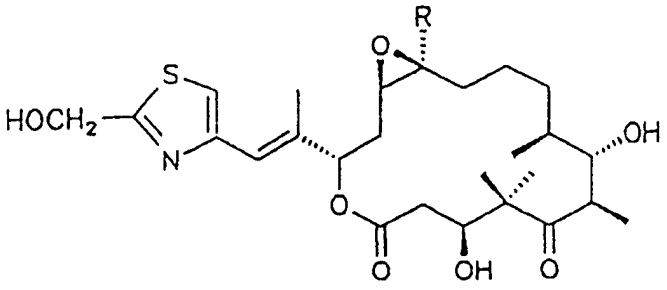
The present invention concerns the epothilone, especially epothilone C (R = hydrogen) and epothilone D (R = methyl) of formula (I), as well as epothilone E (R = hydrogen) and epothilone F (R = methyl) of formula (II), the production process and their application for producing therapeutic agents, including cytostatic agents, as well as phytosanitary agents.



(I)

(57) Zusammenfassung

Die vorliegende Erfindung betrifft Epothilone, insbesondere Epothilon C (R = Wasserstoff) und Epothilon D (R = Methyl) der Formel (I) sowie Epothilon E (R = Wasserstoff) und Epothilon F (R = Methyl) der Formel (II), deren Herstellung, sowie deren Verwendung zur Herstellung von therapeutischen, insbesondere cytostatischen Mitteln sowie Mitteln für den Pflanzenschutz.



(II)

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AL	Albanien	**ES**	Spanien	**LS**	Lesotho	**SI**	Slowenien
AM	Armenien	**FI**	Finnland	**LT**	Litauen	**SK**	Slowakei
AT	Österreich	**FR**	Frankreich	**LU**	Luxemburg	**SN**	Senegal
AU	Australien	**GA**	Gabun	**LV**	Lettland	**SZ**	Swasiland
AZ	Aserbaidschan	**GB**	Vereinigtes Königreich	**MC**	Monaco	**TD**	Tschad
BA	Bosnien-Herzegowina	**GE**	Georgien	**MD**	Republik Moldau	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagaskar	**TJ**	Tadschikistan
BE	Belgien	**GN**	Guinea	**MK**	Die ehemalige jugoslawische Republik Mazedonien	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Griechenland			**TR**	Türkei
BG	Bulgarien	**HU**	Ungarn	**ML**	Mali	**TT**	Trinidad und Tobago
BJ	Benin	**IE**	Irland	**MN**	Mongolei	**UA**	Ukraine
BR	Brasilien	**IL**	Israel	**MR**	Mauretanien	**UG**	Uganda
BY	Belarus	**IS**	Island	**MW**	Malawi	**US**	Vereinigte Staaten von Amerika
CA	Kanada	**IT**	Italien	**MX**	Mexiko		
CF	Zentralafrikanische Republik	**JP**	Japan	**NE**	Niger	**UZ**	Usbekistan
CG	Kongo	**KE**	Kenia	**NL**	Niederlande	**VN**	Vietnam
CH	Schweiz	**KG**	Kirgisistan	**NO**	Norwegen	**YU**	Jugoslawien
CI	Côte d'Ivoire	**KP**	Demokratische Volksrepublik Korea	**NZ**	Neuseeland	**ZW**	Zimbabwe
CM	Kamerun			**PL**	Polen		
CN	China	**KR**	Republik Korea	**PT**	Portugal		
CU	Kuba	**KZ**	Kasachstan	**RO**	Rumänien		
CZ	Tschechische Republik	**LC**	St. Lucia	**RU**	Russische Föderation		
DE	Deutschland	**LI**	Liechtenstein	**SD**	Sudan		
DK	Dänemark	**LK**	Sri Lanka	**SE**	Schweden		
EE	Estland	**LR**	Liberia	**SG**	Singapur		

EPOTHILONE C, D, E UND F, DEREN HERSTELLUNG UND DEREN VERWENDUNG ALS CYTOSTATISCHE MITTEL BZW. ALS PFLANZENSCHUTZMITTEL

Die vorliegende Erfindung betrifft Epothilone C, D, E und F, deren Herstellung sowie deren Verwendung zur Herstellung von therapeutischen Mitteln und Mitteln für den Pflanzenschutz.

Epothilone C und D

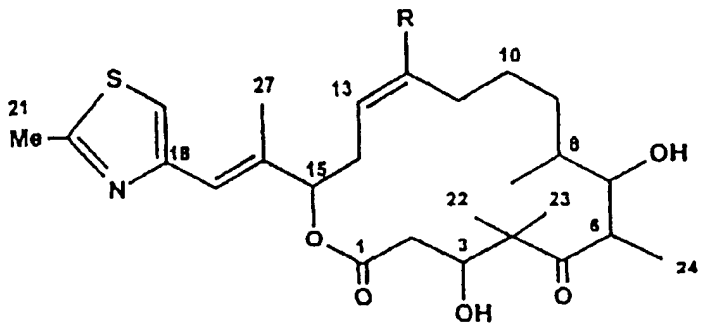
Gemäß einer Ausführungsform betrifft die Erfindung Epothilone [C und D], die dadurch gewinnbar sind, daß man

(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert,
(b) das Adsorberharz von der Kultur abtrennt und mit einem Wasser/Methanol-Gemisch wäscht,
(c) das gewaschene Adsorberharz mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,

(d) das gewonnene Konzentrat mit Ethylacetat extrahiert, den Extrakt einengt und zwischen Methanol und Hexan verteilt,
(e) die methanolische Phase zu einem Raffinat einengt und das Konzentrat an einer Sephadex-Säule fraktioniert,
(f) eine Fraktion mit Stoffwechselprodukten des eingesetzten Mikroorganismus gewinnt,
(g) die gewonnene Fraktion an einer C18-Umkehrphase mir einem Methanol/Wasser-Gemisch chromatographiert und in zeitlicher Reihenfolge
- nach einer ersten Fraktion mit Epothilon A und
- einer zweiten Fraktion mit Epothion B
- eine dritte Fraktion mit einem ersten weiteren Epothilon und
- eine vierte Fraktion mit einem zweiten weiteren Epothilon gewinnt und
(h1) und das Epothilon der ersten weiteren Fraktion und/oder
(h2) das Epothilon der zweiten weiteren Fraktion isoliert.

Ferner betrifft die Erfindung ein Epothilon [C] der Summenformel $C_{26}H_{39}NO_5S$, gekennzeichnet durch das ^1H- und ^{13}C-NMR-Spektrum gemäß Tabelle 1.

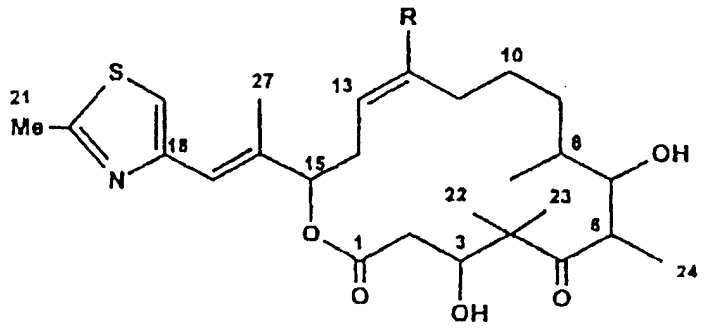
Ferner betrifft die Erfindung Epothilon C der Formel:



Epothilon C R = H

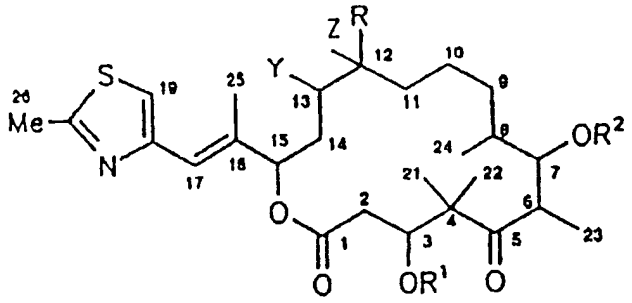
Ferner betrifft die Erfindung Epothilon [D] der Summenformel $C_{27}H_{41}NO_5S$, gekennzeichnet durch das ^1H- und ^{13}C-NMR-Spektrum gemäß Tabelle 1.

Ferner betrifft die Erfindung Epothilon D der Formel:



Epothilon D R = CH_3

Epothilone C und D können zur Herstellung der Verbindungen der folgenden **Formel 1** verwendet werden, wobei zu deren Derivatisierung auf die in WO-A-97/19 086 beschriebenen Derivatisierungsmethoden verwiesen werden kann.



1

In der vorstehenden **Formel 1** bedeuten:

R = H, C_{1-4}-Alkyl;
R^1, R^2, R^3, R^4, R^5 = H, C_{1-6}-Alkyl,

C_{1-6}-Acyl-Benzoyl,
C_{1-4}-Trialkylsilyl,
Benzyl,
Phenyl,
C_{1-6}-Alkoxy-,
C_6-Alkyl-, Hydroxy- und Halogen-substituiertes Benzyl bzw. Phenyl;

wobei auch zwei der Reste R^1 bis R^5 zu der Gruppierung $-(CH_2)_n-$ mit n = 1 bis 6 zusammentreten können und es sich bei den in den Resten enthaltenen Alkyl- bzw. Acylgruppen um gradkettige oder verzweigte Reste handelt;
Y und Z sind entweder gleich oder verschieden und stehen jeweils für Wasserstoff, Halogen, wie F, Cl, Br oder J, Pseudohalogen, wie -NCO, -NCS oder $-N_3$, OH, O-(C_{1-6})-Acyl, O-(C_{1-6})-Alkyl, O-Benzoyl. Y und Z können auch das O-Atom eines Epoxides sein, wobei Epothilon A und B nicht beansprucht werden, oder eine der C-C-Bindungen einer C=C-Doppelbindung bilden.

So kann man die 12,13-Doppelbindung selektiv
- hydrieren, beispielsweise katalytisch oder mit Diimin, wobei man eine Verbindung der **Formel 1** mit Y = Z = H erhält; oder
- epoxidieren, beispielsweise mit Dimethyldioxiran oder einer Persäure, wobei man eine Verbindung der **Formel 1** mit Y mit Z = -O- erhält; oder
- in die Dihalogenide, Dipseudohalogenide oder Diazide umwandeln, wobei man eine Verbindung der **Formel 1** mit Y und Z = Hal, Pseudo-hal oder N_3 erhält.

Epothilone E und F

Gemäß einer weiteren Ausführungsform betrifft die Erfindung einen Biotransformant von Epothilon A, der dadurch gewinnbar ist, daß man

(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert, vom Adsorberharz abtrennt und gegebenenfalls die Gesamtmenge oder einen Teil der abgetrennten Kultur mit einer methanolischen Lösung von Epothilon A versetzt,

(b) die mit Epothilon A versetzte Kultur inkubiert und danach mit Adsorberharz versetzt,

(c) das Adsorberharz von der Kultur abtrennt, mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,

(d) den Rohextrakt zwischen Ethylacetat und Wasser verteilt, die Ethylacetat-Phase abtrennt und zu einem Öl einengt,

(e) das Öl an einer Umkehrphase unter folgenden Bedingungen chromatographiert:

Säulenmaterial:	Nucleosil 100 C-18 7 µm
Säulenmaße:	250 x 16 mm
Laufmittel:	Methanol/Wasser = 60 : 40
Fluß:	10 ml/min

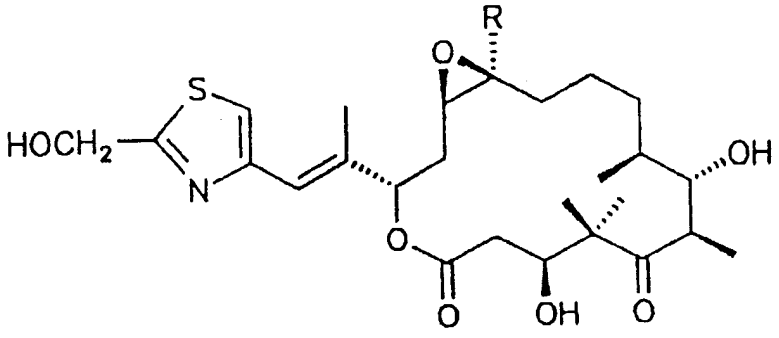
und Fraktionen mit einem Gehalt an Biotransformant, die sich durch UV-Löschung bei 254 nm detektieren lassen, mit einem R_t-Wert von 20 min abtrennt und den Biotransformanten isoliert.

Ferner betrifft die Erfindung einen derartigen Biotransformant von Epothilon A, der dadurch gewinnbar ist, daß man bei Stufe (a) eine Kultur abtrennt, die drei oder vier oder mehr Tage alt ist.

Ferner betrifft die Erfindung einen derartigen Biotransformant von Epothilon A, der dadurch gewinnbar ist, daß man bei Stufe (b) ein oder zwei oder mehr Tage inkubiert.

Ferner betrifft die Erfindung eine Verbindung der Summenformel $C_{26}H_{39}NO_7S$, gekennzeichnet durch folgendes ^{1}H-NMR-Spektrum (300 MHz, $CDCl_3$): delta = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3)

Ferner betrifft die Erfindung eine Verbindung (Epothilon E) der Formel:



Epothilon E R = H

Gemäß einer weiteren Ausführungsform betrifft die Erfindung einen Biotransformant von Epothilon B, der dadurch gewinnbar ist, daß man

(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert, vom Adsorberharz abtrennt und gegebenenfalls die Gesamtmenge oder einen Teil der abgetrennten Kultur mit einer methanolischen Lösung von Epothilon B versetzt,

(b) die mit Epothilon B versetzte Kultur inkubiert und danach mit Adsorberharz versetzt,

(c) das Adsorberharz von der Kultur abtrennt, mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,

(d) den Rohextrakt zwischen Ethylacetat und Wasser verteilt, die Ethylacetat-Phase abtrennt und zu einem Öl einengt,

(e) das Öl an einer Umkehrphase unter folgenden Bedingungen chromatographiert:

Säulenmaterial:	Nucleosil 100 C-18 7 µm
Säulenmaße:	250 x 16 mm
Laufmittel:	Methanol/Wasser = 60 : 40
Fluß:	10 ml/min

und Fraktionen mit einem Gehalt an Biotransformant, die sich durch UV-Löschung bei 254 nm detektieren lassen, mit einem R_t-Wert von 24,5 min abtrennt und den Biotransformanten isoliert.

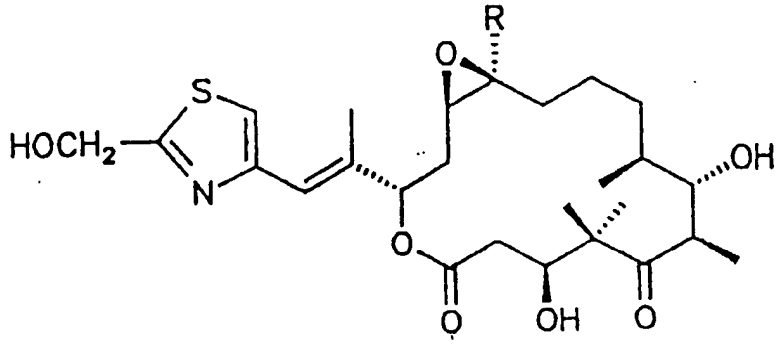
Ferner betrifft die Erfindung einen derartigen Biotransformant von Epothilon B, der dadurch gewinnbar ist, daß man bei Stufe (a) eine Kultur abtrennt, die drei oder vier oder mehr Tage alt ist.

Ferner betrifft die Erfindung einen derartigen Biotransformant von Epothilon B, der dadurch gewinnbar ist, daß man bei Stufe (b) ein oder zwei oder mehr Tage inkubiert.

Ferner betrifft die Erfindung eine Verbindung der Summenformel $C_{27}H_{41}NO_7S$, gekennzeichnet durch folgendes ^{1}H-NMR-Spektrum (300 MH_z, $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H),

7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Ferner betrifft die Erfindung eine Verbindung (Epothilon F) der Formel:



Epothilon F R = CH_3

Herstellung und Mittel

Die erfindungsgemäßen Verbindungen bzw. Epothilone sind mit den vorstehend angeführten Maßnahmen gewinnbar.

Die Erfindung betrifft ferner Mittel für den Pflanzenschutz in Landwirtschaft, Forstwirtschaft und/oder Gartenbau, bestehend aus einem oder mehreren der vorstehend aufgeführten Epothilone C, D, E und F bzw. bestehend aus einem oder mehreren der vorstehend aufgeführten Epothilone neben einem oder mehreren üblichen Träger(n) und/oder Verdünnungsmittel(n).

Schließlich betrifft die Erfindung therapeutische Mittel, bestehend aus einer oder mehreren der vorstehend aufgeführten Verbindungen oder einer oder mehreren der vorstehend aufgeführten Verbindungen neben einem oder mehreren üblichen Träger(n) und/oder Verdünnungsmittel(n). Diese Mittel können insbesondere cytotoxische Aktivitäten zeigen und/oder Immunsuppression bewirken

und/oder zur Bekämpfung maligner Tumore eingesetzt werden, wobei sie besonders bevorzugt als Cytostatika verwendbar sind.

Die Erfindung wird im folgenden durch die Beschreibung von einigen ausgewählten Ausführungsbeispielen näher erläutert und beschrieben.

Beispiele

Beispiel 1:
Epothilone C und D

A. Produktionsstamm und Kulturbedingungen entsprechend dem Epothilon Basispatent DE-B-41 38 042.

B. Produktion mit DSM 6773

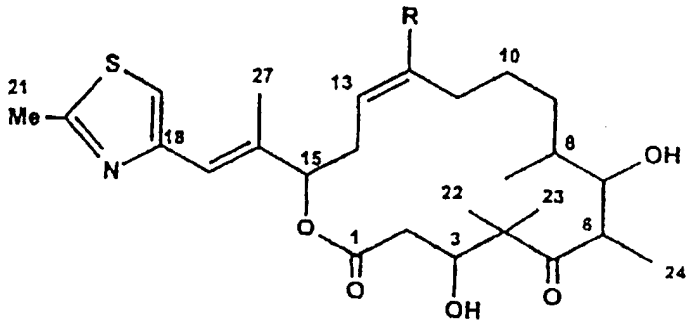
75 l Kultur werden wie im Basispatent beschrieben angezogen und zum Animpfen eines Produktionsfermenters mit 700 l Produktionsmedium aus 0.8 % Stärke, 0.2 % Glukose, 0.2 % Soyamehl, 0.2 % Hefeextrakt, 0.1 % $CaCl_2 \times 2H_2O$, 0.1 % $MgSO_4 \times 7H_2O$, 8 mg/l Fe-EDTA, pH = 7.4 und optional 15 l Adsorberharz Amberlite XAD-16 verwendet. Die Fermentation dauert 7 - 10 Tage bei 30 C, Belüftung mit 0.1 NL/m^3. Durch Regulierung der Drehzahl wird der pO_2 bei 30 % gehalten.

C. Isolierung

Das Adsorberharz wird mit einem 0.7 m^2, 100 mesh Prozeßfilter von der Kultur abgetrennt und durch Waschen mit 3 Bettvolumen Wasser/Methanol 2:1 von polaren Begleitstoffen befreit. Durch Elution mit 4 Bettvolumen Methanol wird ein Rohextrakt gewonnen, der i. Vak. bis zum Auftreten der Wasserphase eingedampft wird.

Diese wird dreimal mit dem gleichen Volumen Ethylacetat extrahiert. Eindampfen der organischen Phase ergibt 240 g Rohextrakt, der zwischen Methanol und Heptan verteilt wird, um lipophile Begleitstoffe abzutrennen. Aus der Methanolphase werden durch Eindampfen i. Vak. 180 g Raffinat gewonnen, das in drei Portionen über Sephadex LH-20 (Säule 20 x 100 cm, 20 ml/min Methanol) fraktioniert wird. Die Epothilone sind in der mit 240 - 300 min Retentionszeit eluierten Fraktion von insgesamt 72 g enthalten. Zur Trennung der Epothilone wird in drei Portionen an Lichrosorb RP-18 (15 µm, Säule 10 x 40 cm, Laufmittel 180 ml/min Methanol/Wasser 65:35) chromatographiert. Nach Epothilon A und B werden mit R_t = 90-95 min Epothilon C und 100-110 min Epothilon D eluiert und nach Eindampfen i. Vak. in einer Ausbeute von jeweils 0.3 g als farblose Öle gewonnen.

D. Physikalische Eigenschaften



Epothilon C R = H
Epothilon D R = CH_3

Epothilon C

$C_{26}H_{39}NO_5S$ [477]
ESI-MS: (positiv Ionen): 478.5 für $[M+H]^+$

1H und 13C siehe NMR-Tabelle

DC:R_f = 0,82
DC-Alufolie 60 F 254 Merck, Laufmittel: Dichlormethan/Methanol = 9:1
Detektion: UV-Löschung bei 254 nm. Ansprühen mit Vanillin-Schwefelsäure-Reagenz, blau-graue Anfärbung beim Erhitzen auf 120 °C.

HPLC:R_t = 11,5 min
Säule: Nucleosil 100 C-18 7µm, 125 x 4 mm
Laufmittel: Methanol/Wasser = 65:35
Fluß: 1ml/min
Detection: Diodenarray

Epothilon D

$C_{27}H_{41}NO_5S$ [491]
ESI-MS: (positiv Ionen): 492,5 für $[M+H]^+$

1H und 13C siehe NMR-Tabelle

DC:R_f = 0,82
DC-Alufolie 60 F 254 Merck, Laufmittel: Dichlormethan/Methanol = 9:1
Detektion: UV-Löschung bei 254 nm. Ansprühen mit Vanillin-Schwefelsäure-Reagenz, blau-graue Anfärbung beim Erhitzen auf 120 °C.

HPLC:R_t = 15,3 min
Säule: Nucleosil 100 C-18 7µm, 125 x 4 mm
Laufmittel: Methanol/Wasser = 65:35
Fluß: 1ml/min
Detection: Diodenarray

Tabelle 1: ^{1}H- und ^{13}C-NMR Daten von Epothilon C und Epothilon D in [D_6] DMSO bei 300 MHz

	Epothilon C			Epothilon D		
H-Atom	δ (ppm)	C-Atom	δ (ppm)	δ (ppm)	C-Atom	δ (ppm)
		1	170.3		1	170.1
2-Ha	2.38	2	38.4	2.35	2	39.0
2-Hb	2.50	3	71.2	2.38	3	70.8
3-H	3.97	4	53.1	4.10	4	53.2
3-OH	5.12	5	217.1	5.08	5	217.4
6-H	3.07	6	45.4	3.11	6	44.4
7-H	3.49	7	75.9	3.48	7	75.5
7-OH	4.46	8	35.4	4.46	8	36.3
8-H	1.34	9	27.6	1.29	9	29.9
9-Ha	1.15	10	30.0	1.14	10	25.9
9-Hb	1.40	11	27.6	1.38	11	31.8*
10-Ha	1.15*	12	124.6	1.14*	12	138.3
10-Hb	1.35*	13	133.1	1.35*	13	120.3
11-Ha	1.90	14	31.1	1.75	14	31.6*
11-Hb	2.18	15	76.3	2.10	15	76.6
12-H	5.38**	16	137.3		16	137.2
13-H	5.44**	17	119.1	5.08	17	119.2
14-Ha	2.35	18	152.1	2.30	18	152.1
14-Hb	2.70	19	117.7	2.65	19	117.7
15-H	5.27	20	164.2	5.29	20	164.3
17-H	6.50	21	18.8	6.51	21	18.9
19-H	7.35	22	20.8	7.35	22	19.7
21-H_3	2.65	23	22.6	2.65	23	22.5
22-H_3	0.94	24	16.7	0.90	24	16.4
23-H_3	1.21	25	18.4	1.19	25	18.4
24-H_3	1.06	27	14.2	1.07	26	22.9
25-H_3	0.90			0.91	27	14.1
26-H_3				1.63		
27-H_3	2.10			2.11		

*, ** Zuordnung vertauschbar

Beispiel 2:
Epothilon A und 12,13-Bisepi-epothilon A aus Epothilon C

50 mg Epothilon A werden in 1.5 ml Aceton gelöst und mit 1.5 ml einer 0.07 molaren Lösung von Dimethyldioxiran in Aceton versetzt. Nach 6 Stunden Stehen bei Raumtemperatur wird i. Vak. eingedampft und durch präparative HPLC an Kieselgel (Laufmittel: Methyl-tert.butylether/Petrolether/Methanol 33:66:1) getrennt.

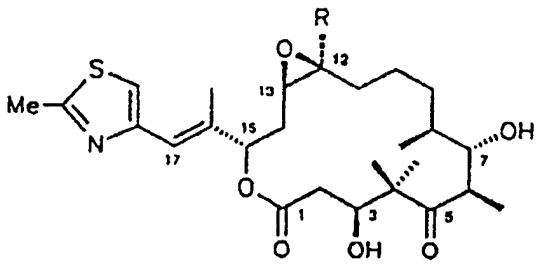
Ausbeute:

25 mg Epothilon A, R_t = 3,5 min (analyt. HPLC, 7 µm, Säule 4 x 250 mm, Laufmittel s. o., Fluß 1.5 ml/min)

und

20 mg 12,13-Bisepi-epothilon A, R_t = 3.7 min, ESI-MS (pos. Ionen)

m/z = 494 $[M+H]^+$
^{1}H-NMR in $[D_4]$ Methanol, ausgewählte Signale: delta = 4.32 (3-H), 3.79 (7-H), 3.06 (12-H), 3.16 (13-H), 5.54 (15-H), 6.69 (17-H), 1.20 (22-H), 1.45 (23-H).



12,13-Bisepi-epothilon A R = H

Beispiel 3:
Epothilon E und F, neue Biotransformationsprodukte der Epothilone A und B.

Produktionsstamm:
Der Produktionsstamm *Sorangium cellulosum* So ce90 wurde im Juli 1985 an der GBF aus einer Bodenprobe von den Ufern des Zambesi isoliert und am 28.10.91 bei der Deutschen Sammlung für Mikroorganismen unter Nr. DSM 6773 hinterlegt.

Die Charakterisierung des Produzenten sowie die Kulturbedingungen sind beschrieben in:
Höfle, G.; N. Bedorf, K. Gerth & H. Reichenbach: Epothilone, deren Herstellungsverfahren sowie sie enthaltende Mittel.
DE 41 38 042 A1, offengelegt am 27. Mai 1993.

Bildung der Epothilone E und E während der Fermentation:

Eine typische Fermentation verläuft folgendermaßen: Ein 100 l Bioreaktor wird mit 60 l Medium (0,8 % Stärke; 0,2 % Glucose; 0,2 % Soyamehl; 0,2 % Hefeextrakt; 0,1 % $CaCl_2 \times 2H_2O$; 0,1 % $MgSO_4 \times 7H_2O$; 8 mg/l Fe-EDTA; pH 7,4) gefüllt. Zusätzlich werden 2 % Adsorberharz (XAD-16, Rohm & Haas) zugegeben. Das Medium wird durch Autoklavieren (2 Std., 120 °C) sterilisiert. Beimpft wird mit 10 l einer im gleichen Medium (zusätzlich 50 mM HEPES-Puffer pH 7,4) im Schüttelkolben angezogenen Vorkultur (160 Upm, 30 °C). Fermentiert wird bei 32 °C mit einer Rührergeschwindigkeit von 500 Upm und einer Belüftung von 0,2 Nl pro m^3 und Std, der pH Wert wird durch Zugabe von KOH bei 7,4 gehalten. Die Fermentation dauert 7 bis 10 Tage. Die gebildeten Epothilone werden während der Fermentation kontinuierlich an das Adsorberharz gebunden. Nach Abtrennen der Kulturbrühe (z. B. durch Absieben in einem Prozeßfilter) wird das Harz mit 3 Bettvolumen Wasser gewa-

schen und mit 4 Bettvolumen Methanol eluiert. Das Eluat wird zur Trockne eingeengt und in 700 ml Methanol aufgenommen.

HPLC-Analyse des XAD-Eluates:

Gegenüber dem Ausgangsvolumen des Reaktors (70 l) ist das Eluat 100:1 konzentriert. Die Analyse wird durchgeführt mit einer HPLC Anlage 1090 der Fa. Hewlett Packard. Zur Trennung der Inhaltstoffe wird eine Microbore Säule (125/2 Nucleosil 120-5 C_{18}) der Fa. Machery-Nagel (Düren) verwendet. Eluiert wird mit einem Gradienten aus Wasser/Acetonitril von anfänglich 75:25 bis zu 50:50 nach 5,5 Minuten. Dieses Verhältnis wird bis zur 7. Minute gehalten, um dann bis zur 10. Minute auf 100 % Acetonitril anzusteigen.

Gemessen wird bei einer Wellenlänge von 250 nm und einer Bandbreite von 4 nm. Die Dioden Array Spektren werden im Wellenlängenbereich von 200 bis 400 nm gemessen. Im XAD-Eluat fallen zwei neue Substanzen mit R_t 5,29 und R_t 5,91 auf, deren Adsorptionsspektren mit denen von Epothilonen A bzw. B identisch sind (Abb. 1; E entspricht A, F entspricht B). Diese Substanzen werden unter den gegebenen Fermentationsbedingungen nur in Spuren gebildet.

Biotransformation von Epothilon A und B zu Epothilon E und F:

Für die gezielte Biotransformation wird eine 4 Tage alte, mit Adsorberharz gehaltene 500 ml Kultur von So ce90 verwendet. Von dieser werden 250 ml unter Zurücklassen des XAD in einen sterilen 1 l Erlenmeyerkolben überführt. Danach wird eine methanolische Lösung einer Mischung von insgesamt 36 mg Epothilon A und 14 mg Epothilon B zugegeben und der Kolben für zwei Tage bei 30 °C und 200 Upm auf einer Schütteltruhe inkubiert. Die Bildung der Epothilone E und F wird direkt aus 10 µl des zentrifugierten Kulturüberstands analysiert (Abb. 2). Die Umwandlung erfolgt nur

in Gegenwart der Zellen und ist abhängig von der eingesetzten Zelldichte und der Zeit. Eine Kinetik der Umwandlung ist für Epothilon A in **Abb.** 3 dargestellt.

Isolierung von Epothilon E und F

Zur Isolierung von Epothilon E und F werden drei Schüttelkolbenansätze aus der Biotransformation (s. o.) vereinigt und 1 h mit 20 ml XAD-16 geschüttelt. Das XAD wird durch Absieben gewonnen und mit 200 ml Methanol eluiert. Das Eluat wird i. Vak. zu 1.7 g Rohextrakt eingedampft. Dieser wird zwischen 30 ml Ethylacetat und 100 ml Wasser verteilt. Aus der Ethylacetatphase werden beim Eindampfen i. Vak. 330 mg eines öligen Rückstandes erhalten, die in fünf Läufen über eine 250 x 20 mm RP-18 Säule chromatographiert werden (Laufmittel: Methanol/Wasser 58:42, Detektion 254 nm).

Ausbeute: Epothilon E 50 mg
F 10 mg

Biologische Wirkung von Epothilon E:

In Zellkulturen wurde die Konzentration bestimmt, welche das Wachstum um 50 % reduziert (IC_{50}) und mit den Werten für Epothilon A verglichen.

Zellinie	IC_{50} (ng/ml)	
	Epothilon E	Epothilon A
HeLa. KB-3.1 (human)	5	1
Mausfibroblasten, L929	20	4

Epothilon E

$C_{26}H_{39}HO_7S$ [509]
ESI-MS: (positiv Ionen): 510.3 für $[M+H]^+$

DC: R_f = 0,58
DC-Alufolie 60 F 254 Merck. Laufmittel: Dichlormethan/Methanol = 9:1
Detektion: UV-Löschung bei 254 nm. Ansprühen mit Vanillin-Schwefelsäure-Reagenz, blau-graue Anfärbung beim Erhitzen auf 120 °C

HPLC: R_t = 5,0 min
Säule: Nucleosil 100 C-18 7µm, 250 x 4 mm
Laufmittel: Methanol/Wasser = 60:40
Fluß: 1,2 ml/min
Detektion: Diodenarray

^{1}H-NMR (300 MHz, $CDCl_3$): delta = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3)

Epothilon F

$C_{27}H_{41}NO_7S$ [523]
ESI-MS: (positiv Ionen): 524.5 für $[M+H]^+$

DC: R_f = 0,58
DC-Alufolie 60 F 254 Merck. Laufmittel: Dichlormethan/Methanol = 9:1
Detektion: UV-Löschung bei 254 nm. Ansprühen mit Vanillin-Schwefelsäure-Reagenz, blau-graue Anfärbung beim Erhitzen auf 120 °C.

HPLC: R_t = 5,4 min
Säule: Nucleosil 100 C-18 7µm, 250 x 4 mm
Laufmittel: Methanol/Wasser = 60:40
Fluß: 1,2 ml/min
Detektion: Diodenarray

^{1}H-NMR (300 MH_z, $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Beispiel 4:

Herstellung von Epothilon E und F durch Biotransformation mit *Sorangium cellulosum* So ce90

1) Durchführung der Biotransformation:

Für die Biotransformation wird eine Kultur von *Sorangium cellulosum* So ce90 verwendet, die für vier Tage in Gegenwart von 2 % XAD 16 Adsorberharz (Fa. Rohm und Haas, Frankfurt/M.) bei 30 °C und 160 Upm geschüttelt wurde. Das Kulturmedium hat folgende Zusammensetzung in g/Liter destilliertem Wasser: Kartoffelstärke (Maizena), 8; Glucose (Maizena), 8; entfettetes Sojamehl, 2; Hefeextrakt (Marcor), 2; Ethylendiamintetraessigsäure, Eisen (III) Natrium Salz, 0,008; $MgSO_4$ x 7 H_2O, 1; $CaCl_2$ x 2 H_2O, 1; HEPES 11,5. Der pH-Wert wird vor dem Autoklavieren mit KOH auf 7,4 eingestellt. Das XAD wird durch Sieben über ein Edelstahlsieb (200 µm Maschenweite) von der Kultur abgetrennt. Die Bakterien werden durch Zentrifugation für 10 min bei 10 000 Upm sedimentiert und das Pellet in 1/5 des Kulturüberstandes resuspendiert. Zu der konzentrierten Bakteriensuspension wird nun Epothilon A bzw. Epothilon B in methanolischer Lösung in einer Kon-

zentration von 0,5 g/Liter zugesetzt. Die Kultur wird wie oben beschrieben weiterkultiviert. Zur Analyse der Biotransformation wird zu den gewünschten Zeiten eine 1 ml Probe entnommen, 0,1 ml XAD zugegeben und die Probe für 30 min bei 30 °C geschüttelt. Eluiert wird das XAD mit Methanol. Das Eluat wird zur Trockene eingeengt und in 0,2 ml Methanol wieder aufgenommen. Diese Probe wird über HPLC analysiert.

Abb. 4) Kinetik der Biotransformation von Epothilon A nach Epothilon E

Abb. 5) Kinetik der Biotransformation von Epothilon B nach Epothilon F.

2) Herstellung von Epothilon E durch Biotransformation von 1 g Epothilon A.

Der Stamm *Sorangium cellulosum* So ce90 wird für vier Tage in 8,5 l des obigen Mediums (jedoch ohne XAD Zusatz) in einem 10 Liter Bioreaktor bei 30 °C, einer Drehzahl von 150 Upm und einer Belüftung von 0,1 vvm angezogen.

Anschließend wird die Kultur durch cross flow Filtration auf 3 l eingeengt. Hierzu werden 0,6 m^2 einer Membran mit einer Porengröße von 0,3 μm verwendet.

Die konzentrierte Kultur wird in einen 4 Liter Bioreaktor überführt und eine methanolische Lösung von 1 g Epothilon A in 10 ml Methanol zugegeben. Anschließend wird die Kultur über einen Zeitraum von 21,5 h weiterkultiviert. Die Temperatur beträgt 32 °C, die Rührerdrehzahl 455 Upm und die Belüftung erfolgt mit 6 l/min. Zum Erntezeitpunkt wird 100 ml XAD zugegeben und für 1 h weiterinkubiert. Das XAD wird durch Absieben von den Zellen abgetrennt und erschöpfend mit Methanol eluiert. Das konzentrierte Eluat wird über HPLC analysiert.

Bilanzierung der Biotransformation:

Epothilon A eingesetzt:	1000 mg	= 100 %
Epothilon A nach 21,5 h wiedergefunden:	53,7 mg	= 5,4 %
Epothilon E nach 21,5 h gebildet:	661,4 mg	= 66,1 %
Epothilon A vollständig abgebaut:		= 28,5 %

Versuch 5:

Die erfindungsgemäßen Epothilone wurden mit Zellkulturen (Tabelle 2) und auf Polymerisationsförderung (Tabelle 3) getestet.

Tabelle 2:

Epothilon-Tests mit Zellkulturen

Epothilon	A 493	B 507	C 477	D 491	E 509	F 523
		IC-50 [ng/ml]				
Mausfibroblasten L 929	4	1	100	20	20	1,5
humane Tumorzellinien:						
HL-60 (Leukämie)	0.2	0.2	10	3	1	0,3
K-562 (Leukämie)	0.3	0.3	20	10	2	0,5
U-937 (Lymphom)	0.2	0.2	10	3	1	0,2
KB-3.1 (Cervixkarzinom)	1	0.6	20	12	5	0,5
KB-V1 (Cervixkarzinom multires	0.3	0.3	15	3	5	0,6
A-498 (Nierenkarzinom)	-	1.5	150	20	20	3
A-549 (Lungenkarzinom)	0.7	0.1	30	10	3	0,1

Tabelle 3:

Polymerisationstest mit Epothilonen

Parameter: Zeit bis zur halbmaximalen Polymerisation der Kontrolle

Messung:	w	x	y	z	**Mittel [s]**	**Mittel [%]**
Kontrolle	200	170	180	210	**190**	**100**
Epothilon A	95	60	70	70	**74**	**39**
Epothilon B		23	25	30	**26**	**14**
Epothilon C	125	76	95	80	**94**	**49**
Epothilon D	125	73	120		**106**	**56**
Epothilon E	80	60	50	45	**59**	**31**
Epothilon F	80	40	30	50	**50**	**26**

Standardtest mit 0,9 mg Tubulin/ml und 1 µM Probenkonzentration

Der Polymerisationstest ist ein in vitro Test mit gereinigtem Tubulin aus Schweinehirn. Die Auswertung erfolgt photometrisch. Polymerisationsfördernde Substanzen wie die Epothilone verkürzen die Zeit, bis zu der halbmaximale Polymerisation erfolgt ist, d. h., je kürzer die Zeit, desto wirksamer die Verbindung. w, x, y und z sind vier unabhängige Versuche, die relative Wirksamkeit ist in der letzten Spalte in % der Kontrolle ausgedrückt; wieder zeigen die niedrigsten Werte die beste Wirksamkeit an. Die Rangliste entspricht ziemlich genau der in Zellkulturen festgestellten.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Gesellschaft für
Biotechnologische
Forschung mbH
Mascheroder Weg 1
3300 Braunschweig

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Gesellschaft für Biotechnologische Address: Forschung mbH Mascheroder Weg 1 3300 Braunschweig	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773 Date of the deposit or of the transfer[1]: 1991-10-28

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1991-10-28 .[2]
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSM DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 1991-11-05

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSM-BP/9 (sole page) 0787

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Gesellschaft für
Biotechnologische
Forschung mbH
Mascheroder Weg 1
3300 Braunschweig

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR So ce 90	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts this microorganism identified under I. above, which was received by it on 1991-10-28 (Date of original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSM-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Adress: Mascheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 1991-11-05

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Patentansprüche

1. Epothilone, dadurch gewinnbar, daß man

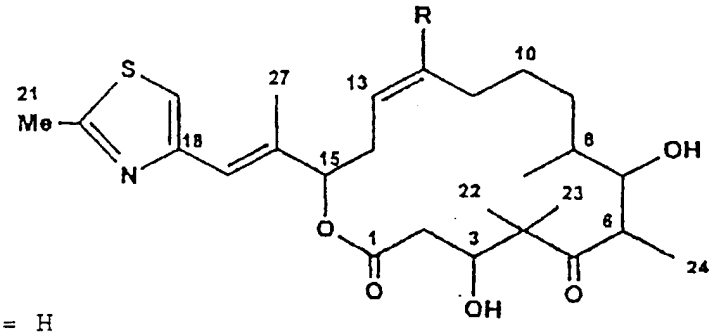
(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert,
(b) das Adsorberharz von der Kultur abtrennt und mit einem Wasser/Methanol-Gemisch wäscht,
(c) das gewaschene Adsorberharz mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,
(d) das gewonnene Konzentrat mit Ethylacetat extrahiert, den Extrakt einengt und zwischen Methanol und Hexan verteilt,
(e) die methanolische Phase zu einem Raffinat einengt und das Konzentrat an einer Sephadex-Säule fraktioniert,
(f) eine Fraktion mit Stoffwechselprodukten des eingesetzten Mikroorganismus gewinnt,
(g) die gewonnene Fraktion an einer C18-Umkehrphase mir einem Methanol/Wasser-Gemisch chromatographiert und in zeitlicher Reihenfolge
- nach einer ersten Fraktion mit Epothilon A und
- einer zweiten Fraktion mit Epothion B

- eine dritte Fraktion mit einem ersten weiteren Epothilon und
- eine vierte Fraktion mit einem zweiten weiteren Epothilon gewinnt und

(h1) und das Epothilon der ersten weiteren Fraktion und /oder
(h2) das Epothilon der zweiten weiteren Fraktion isoliert.

2. Epothilon der Summenformel $C_{26}H_{39}NO_5S$, **gekennzeichnet** durch das 1H- und 13C-NMR-Spektrum gemäß Tabelle 1.

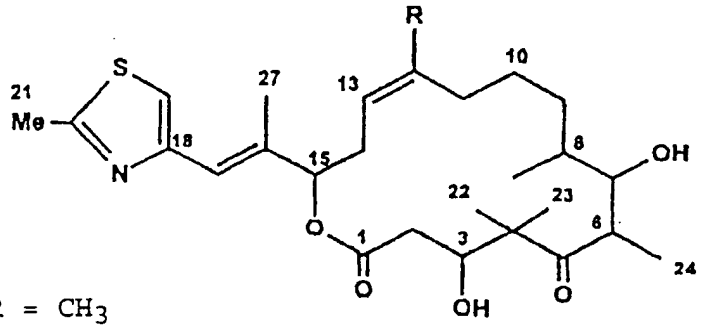
3. Epothilon C der Formel:



Epothilon C R = H

4. Epothilon der Summenformel $C_{27}H_{41}NO_5S$, **gekennzeichnet** durch das ^{1}H- und ^{13}C-NMR-Spektrum gemäß Tabelle 1.

5. Epothilon D der Formel:



Epothilon D R = CH_3

6. Biotransformant von Epothilon A, dadurch gewinnbar, daß man

(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert, vom Adsorberharz abtrennt und gegebenenfalls die Gesamtmenge oder einen Teil der abgetrennten Kultur mit einer methanolischen Lösung von Epothilon A versetzt,

(b) die mit Epothilon A versetzte Kultur inkubiert und danach mit Adsorberharz versetzt,

(c) das Adsorberharz von der Kultur abtrennt, mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,

(d) den Rohextrakt zwischen Ethylacetat und Wasser verteilt, die Ethylacetat-Phase abtrennt und zu einem Öl einengt,

(e) das Öl an einer Umkehrphase unter folgenden Bedingungen chromatographiert:

Säulenmaterial:	Nucleosil 100 C-18 7 µm
Säulenmaße:	250 x 16 mm
Laufmittel:	Methanol/Wasser = 60 : 40
Fluß:	10 ml/min

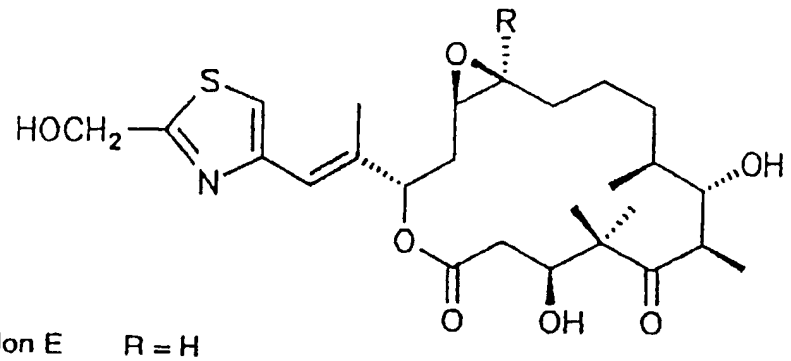
und Fraktionen mit einem Gehalt an Biotransformant, die sich durch UV-Löschung bei 254 nm detektieren lassen, mit einem R_t-Wert von 20 min abtrennt und den Biotransformanten isoliert.

7. Biotransformant von Epothilon A nach Anspruch 6, dadurch gewinnbar, daß man bei Stufe (a) eine Kultur abtrennt, die drei oder vier oder mehr Tage alt ist.

8. Biotransformant von Epothilon A nach Anspruch 6 oder 7, dadurch gewinnbar, daß man bei Stufe (b) ein oder zwei oder mehr Tage inkubiert.

9. Verbindung der Summenformel $C_{26}H_{39}NO_7S$, **gekennzeichnet** durch folgendes ^1H-NMR-Spektrum (300 MHz, $CDCl_3$): delta = 2.38 ($2\text{-}H_a$), 2.51 ($2\text{-}H_b$), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, $9\text{-}H_2$, $10\text{-}H_2$, $11\text{-}H_2$), 2.89 (12-H), 3.00 (13-H), 1.88 ($14\text{-}H_a$), 2.07 ($14\text{-}H_b$), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 ($21\text{-}H_2$), 1.05 ($22\text{-}H_3$), 1.32 ($23\text{-}H_3$), 1.17 ($24\text{-}H_3$), 0.97 ($25\text{-}H_3$), 2.04 ($27\text{-}H_3$)

10. Verbindung (Epothilon E) der Formel:



Epothilon E R = H

11. Biotransformant von Epothilon B, dadurch gewinnbar, daß man

(a) *Sorangium cellulosum* DSM 6773 in Gegenwart eines Adsorberharzes in an sich bekannter Weise kultiviert, vom Adsorberharz abtrennt und gegebenenfalls die Gesamtmenge oder einen Teil der abgetrennten Kultur mit einer methanolischen Lösung von Epothilon B versetzt,

(b) die mit Epothilon B versetzte Kultur inkubiert und danach mit Adsorberharz versetzt,

(c) das Adsorberharz von der Kultur abtrennt, mit Methanol eluiert und das Eluat zu einem Rohextrakt einengt,

(d) den Rohextrakt zwischen Ethylacetat und Wasser verteilt, die Ethylacetat-Phase abtrennt und zu einem Öl einengt,

(e) das Öl an einer Umkehrphase unter folgenden Bedingungen chromatographiert:

Säulenmaterial:	Nucleosil 100 C-18 7 µm
Säulenmaße:	250 x 16 mm
Laufmittel:	Methanol/Wasser = 60 : 40
Fluß:	10 ml/min

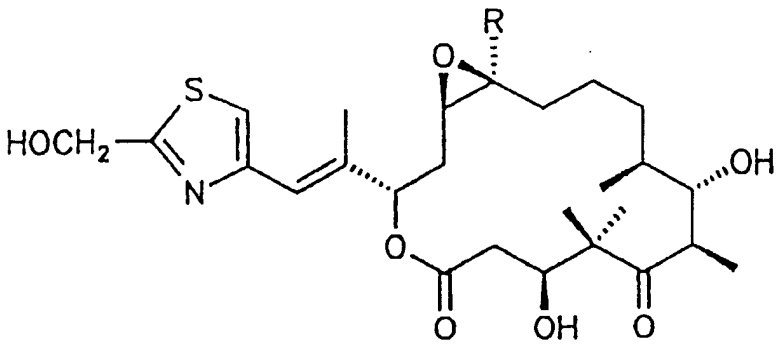
und Fraktionen mit einem Gehalt an Biotransformant, die sich durch UV-Löschung bei 254 nm detektieren lassen, mit einem R_t-Wert von 24,5 min abtrennt und den Biotransformanten isoliert.

12. Biotransformant nach Anspruch 11, dadurch gewinnbar, daß man bei Stufe (a) eine Kultur abtrennt, die drei oder vier oder mehr Tage alt ist.

13. Biotransformant nach Anspruch 11 oder 12, dadurch gewinnbar, daß man bei Stufe (b) ein oder zwei oder mehr Tage inkubiert.

14. Verbindung der Summenformel $C_{27}H_{41}NO_7S$, **gekennzeichnet** durch folgendes ^1H-NMR-Spektrum (300 MH_z, $CDCl_3$): delta = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3)

15. Verbindung (Epothilon F) der Formel:



Epothilon F R = CH_3

16. Mittel für den Pflanzenschutz in der Landwirtschaft und Forstwirschaft und/oder im Gartenbau, bestehend aus einem oder mehreren der Verbindungen gemäß einem der vorangehenden Ansprüche oder einer oder mehreren dieser Verbindungen neben einem oder mehreren üblichen Träger(n) und/oder Verdünnungsmittel(n).

17. Therapeutisches Mittel, insbesondere zum Einsatz als Cytostatikum, bestehend aus einer oder mehreren der Verbindungen nach einem oder mehreren der vorhergehenden Ansprüche oder einer oder mehrerer der Verbindungen nach einem oder mehreren der vorhergehenden Ansprüche neben einem oder mehreren üblichen Träger(n) und/oder Verdünnungsmittel(n).

Fig. 1

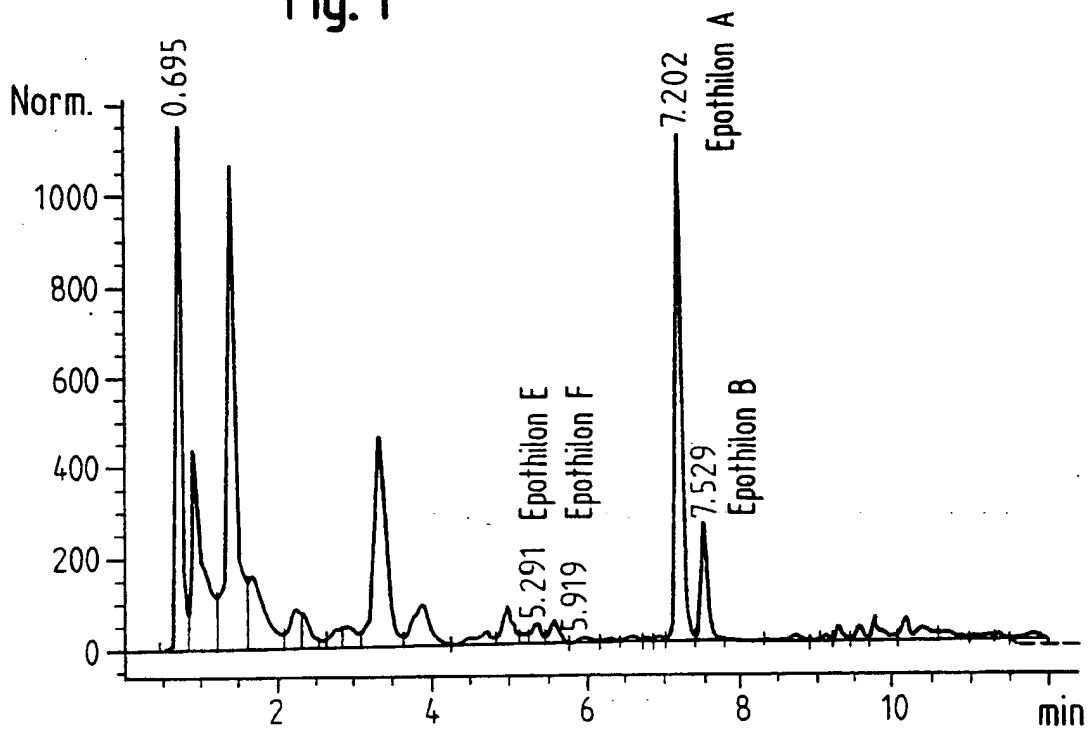

Norm.
0.695
7.202
Epothilon A
Epothilon E
Epothilon F
5.291
5.919
7.529
Epothilon B
1000
800
600
400
200
0
2
4
6
8
10
min

Fig. 2

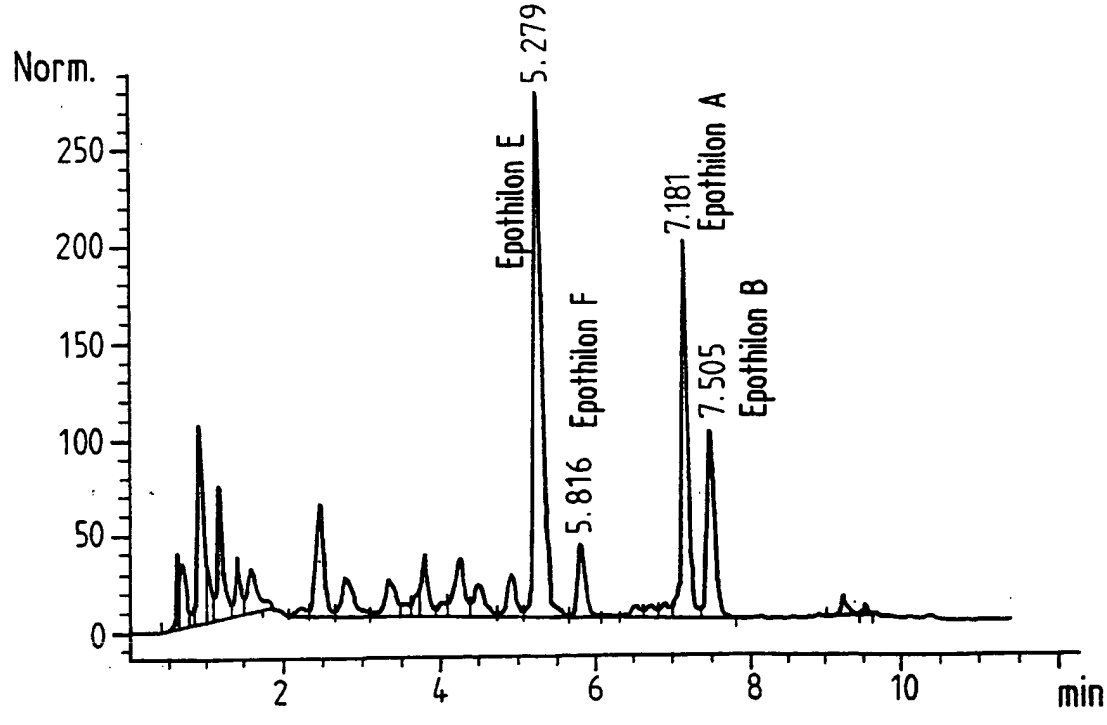

Norm.
5.279
Epothilon E
7.181
Epothilon A
Epothilon F
5.816
7.505
Epothilon B
250
200
150
100
50
0
2
4
6
8
10
min

Fig. 3

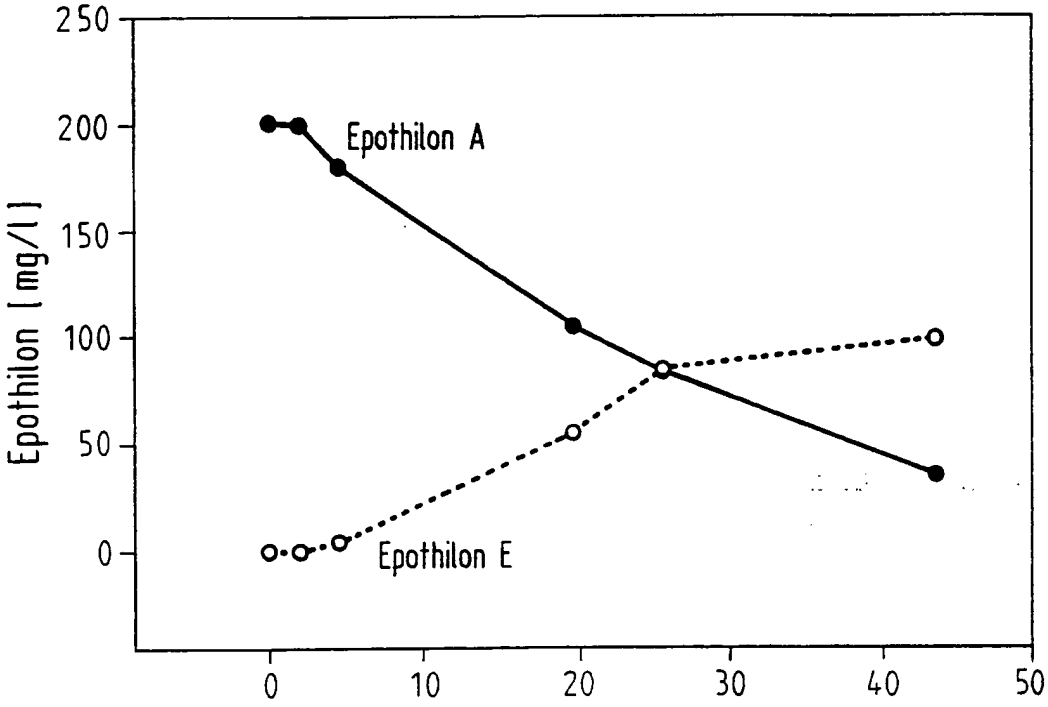

250
200
150
100
50
0
Epothilon [mg/l]
Epothilon A
Epothilon E
0
10
20
30
40
50

Fig. 4

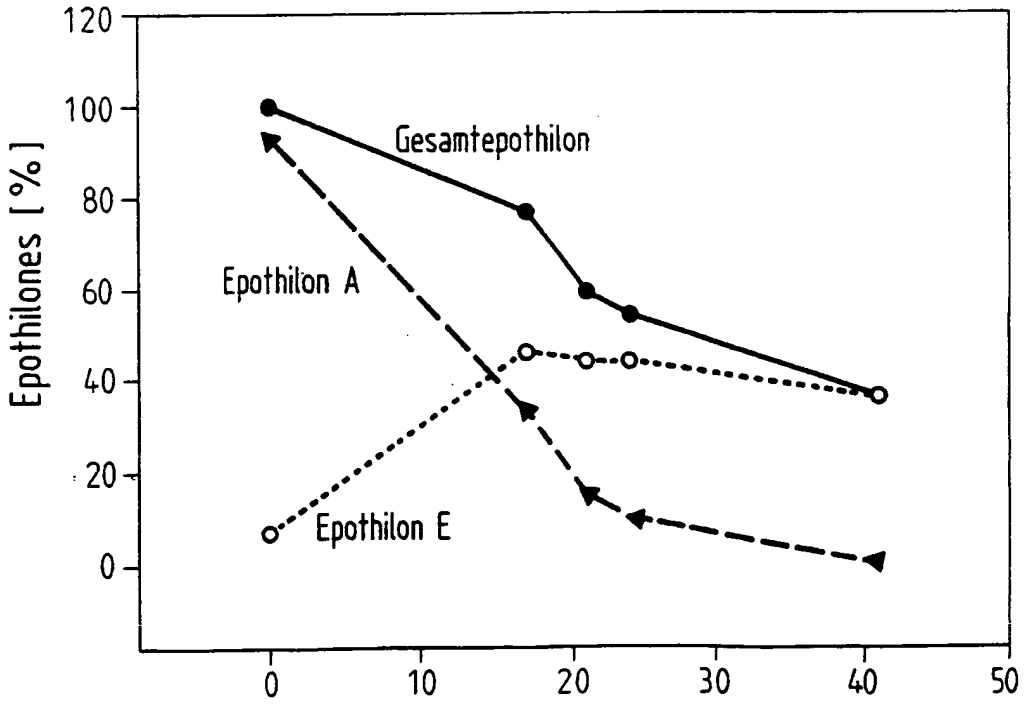
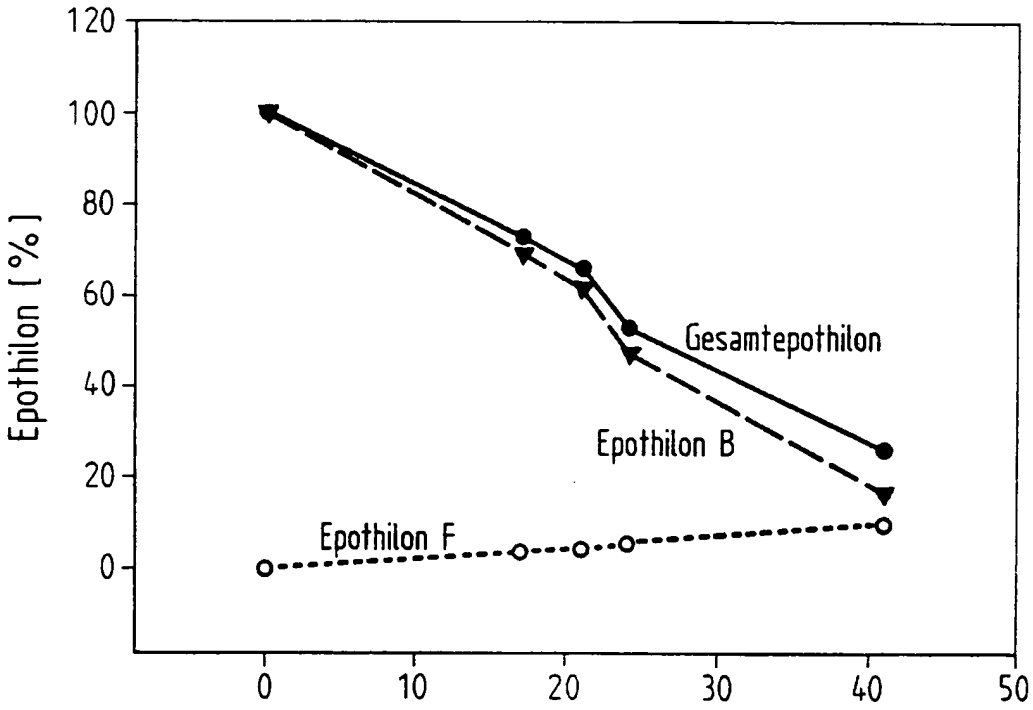

120
100
80
60
40
20
0
Epothilones [%]
Gesamtepothilon
Epothilon A
Epothilon E
0
10
20
30
40
50

Fig. 5


120
100
80
60
40
20
0
Epothilon [%]
0
10
20
30
40
50
Gesamtepothilon
Epothilon B
Epothilon F

INTERNATIONAL SEARCH REPORT

Intern. .nal Application No
PCT/EP 97/06442

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D417/06 C07D493/04 C12P17/08 A01N43/78 A61K31/425
//(C07D493/04,313:00,303:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 10121 A (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) & CIBA GEIGY AG) 27 May 1993 see claims 1,5-8 & DE 41 38 042 A (GBF) 27 May 1993 cited in the application ---	1,16,17
P,X	BALOG A. ET AL.: "Total synthesis of (-)-epothilone A" ANGEWANDTE CHEMIE, INTERNATIONAL EDITION IN ENGLISH, vol. 35, no. 23/24, 3 January 1997, pages 2801-2803, XP002035359 see compound 23; page 2803 --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1998	09. 04. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hartrampf, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr... ...nal Application No
PCT/EP 97/06442

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 19086 A (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF)) 29 May 1997 cited in the application see page 22 - page 26; claims 12,13; example 15	1-5,10, 16,17
E	WO 98 08849 A (NOVARTIS AKTIENGESELLSCHAFT) 5 March 1998 see compounds 19 and 19a, page 32 see claims 2,9	2-5
A	NICOLAOU K.C. ET AL.: "An approach to epothilones based on olefin metathesis" ANGEWANDTE CHEMIE, INTERNATIONAL EDITION IN ENGLISH, vol. 35, no. 20, 4 November 1996, pages 2399-2401, XP002035372	1-17

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interm. ial Application No
PCT/EP 97/06442

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9310121 A	27-05-93	DE 4138042 A	27-05-93
		AU 2943792 A	15-06-93
WO 9719086 A	29-05-97	DE 19542986 A	22-05-97
		DE 19639456 A	26-03-98
WO 9808849 A	05-03-98	DE 19636343 C	23-10-97

INTERNATIONALER RECHERCHENBERICHT

Intern. ,nales Aktenzeichen
PCT/EP 97/06442

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 6 C07D417/06 C07D493/04 C12P17/08 A01N43/78 A61K31/425
//(C07D493/04,313:00,303:00)

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 6 C07D

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 93 10121 A (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) & CIBA GEIGY AG) 27.Mai 1993 siehe Ansprüche 1,5-8 & DE 41 38 042 A (GBF) 27.Mai 1993 in der Anmeldung erwähnt ---	1,16,17
P,X	BALOG A. ET AL.: "Total synthesis of (-)-epothilone A" ANGEWANDTE CHEMIE, INTERNATIONAL EDITION IN ENGLISH, Bd. 35, Nr. 23/24, 3.Januar 1997, Seiten 2801-2803, XP002035359 siehe Verbindung 23; Seite 2803 --- -/--	1-3

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist
"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist
"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist
"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

2

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
27.März 1998	09. 04. 98
Name und Postanschrift der Internationalen Recherchenbehörde Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Bevollmächtigter Bediensteter** Hartrampf, G

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Internales Aktenzeichen
PCT/EP 97/06442

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie°	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	WO 97 19086 A (GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF)) 29.Mai 1997 in der Anmeldung erwähnt siehe Seite 22 - Seite 26; Ansprüche 12,13; Beispiel 15 ---	1-5,10, 16,17
E	WO 98 08849 A (NOVARTIS AKTIENGESELLSCHAFT) 5.März 1998 siehe Verbindungen 19 und 19a, Seite 32 siehe Ansprüche 2,9 ---	2-5
A	NICOLAOU K.C. ET AL.: "An approach to epothilones based on olefin metathesis" ANGEWANDTE CHEMIE, INTERNATIONAL EDITION IN ENGLISH, Bd. 35, Nr. 20, 4.November 1996, Seiten 2399-2401, XP002035372 -----	1-17

2

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internales Aktenzeichen
PCT/EP 97/06442

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO 9310121 A	27-05-93	DE 4138042 A	27-05-93
		AU 2943792 A	15-06-93
WO 9719086 A	29-05-97	DE 19542986 A	22-05-97
		DE 19639456 A	26-03-98
WO 9808849 A	05-03-98	DE 19636343 C	23-10-97

PCT WORLD ORGANIZATION FOR INTELLECTUAL PROPERTY
International Office [SEAL]

INTERNATIONAL PATENT APPLICATION
PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification[6]:
C07D 417/06, 493/04, C12P 17/08, A01N 43/78, A61K 31/425 // (C07D 493/04, 313:00, 303:00)

A1

(11) International Publication Number:
WO 98/22461

(43) International Publication Date:
May 28, 1998 (5/28/98)

(21) International Application No.: PCT/EP97/06442

(22) International Filing Date: November 18, 1997 (11/18/97)

(30) Priority Dates:
196 47 580.5 Nov. 18, 1996 (11/18/96) Germany
197 07 506.1 Feb. 25, 1997 (02/25/97) Germany

(71) Applicant (*for all contracting nations except USA*): GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) [COMPANY FOR BIOTECHNOLOGICAL RESEARCH] [Germany/ Germany], Mascheroder Weg 1, D-38124 Braunschweig (Germany).

(72) Inventor; and

(75) Inventor/applicant (*only for USA*): Hans REICHENBACH [Germany/Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany); Gerhard HOFLE [Germany/ Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany). Klaus GERTH [Germany/Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany). Heinrich STEINMETZ [Germany/Germany] Mascheroder Weg 1, D-38124 Braunschweig (Germany).

(74) Attorney: Hans D. BOETERS et al.; Boeters & Bauer, Bereiteranger 15, D-81541 Munich (Germany).

(81) Contracting nations: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN; ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW); Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE); OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With the International Search Report.
If there are any changes before the final date of the period allowed for changes, publication will be repeated.

(54) Title: EPOTHILONES C, D, E AND F, PRODUCTION PROCESS AND THEIR USE AS CYTOSTATIC AS WELL AS PHYTOSANITARY AGENTS

[Insert (57) Abstract in English] [Insert I, II]

FOR INFORMATIONAL PURPOSES ONLY

Codes used to define PCT countries added to page 1 of the pamphlet of unexamined international applications according to the PCT.

AL	Albania	**ES**	Spain	**LT**	Lithuania	**SN**	Senegal
AM	Armenia	**FI**	Finland	**LU**	Luxembourg	**SZ**	Swaziland
AT	Austria	**FR**	France	**LV**	Latvia	**TD**	Chad
AU	Australia	**GA**	Gabon	**MC**	Monaco	**TG**	Togo
AZ	Azerbaijan	**GB**	Great Britain	**MD**	Moldova	**TJ**	Tajikistan
BA	Bosnia-Herzegovina	**GE**	Georgia	**MG**	Madagascar	**TM**	Turkmenistan
BB	Barbados	**GH**	Ghana	**MK**	Macedonia, former Yugoslavia Republic	**TR**	Turkey
BE	Belgium	**GN**	Guinea	**ML**	Mali	**TT**	Trinidad & Tobago
BF	Burkina Paso	**GR**	Greece	**MN**	Mongolia	**UA**	Ukraine
BG	Bulgaria	**HU**	Hungary	**MR**	Mauritania	**UG**	Uganda
BJ	Benin	**IE**	Ireland	**MW**	Republic of Malawi	**US**	United States of America
BR	Brazil	**IL**	Israel	**MX**	Mexico	**UZ**	Republic of Uzbekistan
BY	Belarus	**IS**	Iceland	**NE**	Niger	**VN**	Vietnam
CA	Canada	**IT**	Italy	**NL**	Netherlands	**YU**	Yugoslavia
CF	Central African Republic	**JP**	Japan	**NO**	Norway	**ZW**	Zimbabwe
CG	People's Republic of Congo	**KE**	Kenya	**NZ**	New Zealand		
CH	Switzerland	**KG**	Kyrgyzstan	**PL**	Poland		
CI	Ivory Coast	**KP**	Democratic People's Republic of Korea	**PT**	Portugal		
CM	Cameroon	**KR**	Republic of Korea	**RO**	Romania		
CN	China	**KZ**	Kazakhstan	**RU**	Russian Federation		
CU	Cuba	**LC**	Saint Lucia	**SD**	Sudan		
CZ	Czech Republic	**LI**	Liechtenstein	**SE**	Sweden		
DE	Germany	**LK**	Sri Lanka	**SG**	Singapore		
DK	Denmark	**LR**	Liberia	**SI**	Slovenia		
EE	Estonia	**LS**	Lesotho	**SK**	Slovak Republic		

EPOTHILONES C, D, E AND F, PRODUCTION PROCESS AND THEIR USE AS CYTOSTATIC AS WELL AS PHYTOSANITARY AGENTS

The invention relates to epothilones C, D, E and F, production and use thereof for production of therapeutic agents and phytosanitary agents.

Epothilones C and D

According to one embodiment, this invention relates to epothilones [C and D], which can be produced by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin by an essentially known method,
(b) separating the adsorber resin from the culture and washing it with a water/methanol mixture,
(c) eluting the washed adsorber resin with methanol and concentrating the eluate to yield a crude extract,
(d) extracting the concentrate thus obtained with ethyl acetate, concentrating the extract and distributing it between methanol and hexane,
(e) concentrating the methanolic phase to yield a raffinate and fractionating the concentrate on a Sephadex column,
(f) obtaining a fraction having metabolic products of the microorganism used,
(g) chromatographing the fraction thus obtained with a methanol/water mixture on a C18 reverse phase and, in chronological order,
- after a first fraction having epothilone A and
- after a second fraction having epothione [sic; epothilone] B,
- obtaining a third fraction having a first additional epothilone, and
- obtaining a fourth fraction having a second additional epothilone, and

(h1) isolating the epothilone of the first additional fraction and/or
(h2) isolating the epothilone of the second additional fraction.

Furthermore, this invention relates to an epothilone [C] of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

This invention also relates to epothilone C of the formula:

[insert]

Epothilone C, R = H

Furthermore, this invention relates to epothilone [D] of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

Furthermore, this invention also relates to epothilone D of the formula:

[insert]

Epothilone D, R = CH_3

Epothilones C and D can be used to produce the compounds of the following formula 1, and for their derivatization, reference can be made to the derivatization methods described in WO-A 97/19 086.

[insert]

In formula 1 above, the following meanings are used:

R = H, C_{1-4} alkyl;
R^1, R^2, R^3, R^4, R^5 = H, C_{1-6} alkyl,
C_{1-6} acyl-benzoyl,
C_{1-4} trialkylsilyl,
benzyl,
phenyl,
C_{1-6} alkoxy,
C_6 alkyl-, hydroxy- and halogen-
substituted benzyl or phenyl;
where two of the R^1 through R^5 groups may also be combined to form the $-(CH_2)_n-$ group, where n = 1 to 6, and the alkyl and/or acyl groups contained in the groups may be linear or branched; Y and Z are either the same or different, each standing for hydrogen, halogen, e.g., F, Cl, Br or I, pseudohalogen, e.g., -NCO, -NCS or $-N_3$, OH, O-(C_{1-6})-acyl, O-(C_{1-6})-alkyl, O-benzyl. Y and Z may also denote the O atom of an epoxy, in which case epothilone A and B are not claimed here, or they may form one of the C-C bonds of a C=C double bond.

Thus the 12, 13-double bond may be selectively:
- hydrogenated, e.g., catalytically or with diimine, yielding a compound of formula 1 where Y = Z = H; or
- epoxidized, e.g., with dimethyldioxirane or a peracid, yielding a compound of formula 1 where Y and Z = -O-; or
- converted to the dihalides, dipseudohalides or diazides, yielding a compound of formula 1, where Y and Z = Hal, pseudo-Hal or N_3.

Epothilones E and F

According to another embodiment, this invention relates to a biotransformant of epothilone A obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone A.

(b) incubating the culture mixed with epothilone A and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting with methanol and concentrating the eluate to form a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to yield an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 20 min and isolating the biotransformant.

Furthermore, this invention relates to such a biotransformant as epothilone A, which is obtainable by separating at step (a) a culture, which is three or four or more days old.

Furthermore, this invention relates to such a biotransformant of epothilone A, which is obtainable by incubating the culture for one or two or more days in step (b).

Furthermore, this invention relates to a compound of the empirical formula $C_{26}H_{39}NO_7S$ characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

Furthermore, this invention relates to a compound (epothilone E) of the formula:

[insert]

Epothilone E. R = H

According to another embodiment, this invention relates to a biotransformant of epothilone B, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone B,

(b) incubating the culture mixed with epothilone B and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting with methanol and concentrating the eluate to form a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to yield an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 mL/min

and separating the fractions which contain biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 24.5 min and isolating the biotransformant.

Furthermore, this invention relates to such a biotransformant as epothilone B, which is obtainable by incubating a culture which is three or four or more days old in step (a).

Furthermore, this invention relates to such a biotransformant of epothilone B, which is obtainable by incubating the culture for one or two or more days in step (b).

Furthermore, this invention relates to a compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^{1}H-NMR spectral data (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Furthermore, this invention relates to a compound (epothilone F) of the formula:

[insert]

Epothilone F, R = CH_3

Synthesis and agents

The compounds according to this invention, i.e., epothilones, are obtainable by the measures described above.

This invention also relates to phytosanitary agents for use in agriculture, forestry and/or gardening, consisting of one or more of the epothilones C, D, E and F listed above and/or consisting of one or more of the epothilones listed above plus one or more conventional vehicle(s) and/or diluent(s).

Finally, this invention relates to therapeutic agents consisting of one or more of the compounds listed above or one or more of the compounds listed above plus one or more conventional vehicle(s) and/or diluent(s). These agents may have cytotoxic activities in particular and/or may induce immunosuppression and/or be used to combat malignant tumors, but they can be used especially preferably as cytostatics.

This invention is explained in greater detail below through the description of a few selected exemplary embodiments.

Examples

Example 1:

Epothilones C and D

A. Production strain and culture conditions according to the epothilone basic patent, German Patent 41 38 042 B.

B. Production with DSM 6773

75 liters of culture are cultured as described in the basic patent and used in production fermenter to inoculate 700 L of production medium consisting of 0.8% starch, 0.2% glucose, 0.2% soybean meal, 0.2% yeast extract, 0.1% $CaCl_2$ x $2H_2O$, 0.1% $MgSO_4$ x $7H_2O$, 8 mg/L Fe-EDTA, pH = 7.4 and optionally 15 L of Amberlite XAD-16 adsorber resin. The fermentation lasts for seven to ten days at 30°C, aeration rate 0.1 L [STP]/m^3. The pO_2 is kept at 30% by regulating the rotational speed.

C. Isolation

The adsorber resin is separated from the culture with a 100 mesh process filter (0.7 m^2) and freed of polar impurities by washing with three bed volumes of water/methanol 2:1. By elution with four bed volumes of methanol, a crude extract is obtained and then evaporated *in vacuo* until the aqueous phase appears. This phase is extracted three times with an equal volume of ethyl acetate. Evaporating the organic phase yields 240 g crude extract, which is distributed between methanol and heptane to separate the lipophilic impurities. By evaporating *in vacuo*, 180 g raffinate is obtained from the methanol phase and then is fractionated in three portions over Sephadex LH-20 (column 20 × 100 cm, 20 mL/min methanol). The epothilones are contained in the fraction eluted with a retention time of 240 to 300 minutes, this fraction amounting to a total of 72 g. To separate the epothilones, this fraction is chromatographed in three portions on Lichrosorb RP-18 (15 µm, column 10 × 40 cm, eluent 180 mL/min methanol/water 65:35). After epothilones A and B, epothilone C is eluted with R_t = 90 to 95 min, and epothilone D is eluted with R_t = 100 to 110 min. After evaporating *in vacuo*, each is obtained as a colorless oil in a yield of 0.3 g.

D. Physical properties

[insert]

Epothilone C, R = H
Epothilone D, R = CH_3

Epothilone C

$C_{26}H_{39}NO_5S$ [477]
ESI-MS (positive ions): 478.5 for $[M + H]^+$

^1H and ^{13}C: see NMR table.

TLC: R_f = 0.82
TLC aluminum foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.

HPLC: R_t = 11.5 min
Column: Nucleosil 100 C-18 7µm, 125 × 4 mm
Eluent: methanol/water = 65:35
Flow rate: 1 mL/min
Detection: diode array

Epothilone D

$C_{27}H_{41}NO_5S$ [491]
ESI-MS (positive ions): 492.5 for $[M + H]^+$

^1H and ^{13}C: see NMR table.

TLC: R_f = 0.82
TLC aluminum foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.
HPLC: R_t = 15.3 min
Column: Nucleosil 100 C-18 7µm, 125 × 4 mm
Eluent: methanol/water = 65:35
Flow rate: 1 mL/min
Detection: diode array

Table 1. ^{1}H and ^{13}C NMR data on epothilone C and epothilone D in [D_6] DMSO at 300 MHz.

H atom	Epothilone C			Epothilone D		
	δ (ppm)	C atom	δ (ppm)	δ (ppm)	C atom	δ (ppm)
		1	170.3		1	170.1
2-Ha	2.38	2	38.4	2.35	2	39.0
2-Hb	2.50	3	71.2	2.38	3	70.8
3-H	3.97	4	53.1	4.10	4	53.2
3-OH	5.12	5	217.1	5.08	5	217.4
6-H	3.07	6	45.4	3.11	6	44.4
7-H	3.49	7	75.9	3.48	7	75.5
7-OH	4.46	8	35.4	4.46	8	36.3
8-H	1.34	9	27.6	1.29	9	29.9
9-Ha	1.15	10	30.0	1.14	10	25.9
9-Hb	1.40	11	27.6	1.38	11	31.8*
10-Ha	1.15*	12	124.6	1.14*	12	
10-Hb	1.35*	13	133.1	1.35*	13	138.3
11-Ha	1.90	14	31.1	1.75	14	120.3
11-Hb	2.18	15	76.3	2.10	15	31.6*
12-H	5.38**	16	137.3		16	76.6
13-H	5.44**	17	119.1	5.08	17	137.2
14-Ha	2.35	18	152.1	2.30	18	119.2
14-Hb	2.70	19	117.7	2.65	19	152.1
15-H	5.27	20	164.2	5.29	20	117.7
17-H	6.50	21	18.8	6.51	21	164.3
19-H	7.35	22	20.8	7.35	22	18.9
21-H_3	2.65	23	22.6	2.65	23	19.7
22-H_3	0.94	24	16.7	0.90	24	22.5
23-H_3	1.21	25	18.4	1.19	25	16.4
24-H_3	1.06	27	14.2	1.07	26	18.4
25-H_3	0.90			0.91	27	22.9
26-H_3				1.63		14.1
27-H_3	2.10			2.11		

*, ** Assignment interchangeable

Example 2:

Epothilone A and 12, 13-bisepi-epothilone A from epothilone C

50 mg epothilone A is dissolved in 1.5 mL acetone and mixed with 1.5 mL of a 0.07 molar solution of dimethyldioxirane in acetone. After standing for six hours at room temperature, the mixture is evaporated *in vacuo* and separated by preparative HPLC on silica gel (eluent: methyl-tert-butyl ether/petroleum ether/methanol 33:66:1).

Yield:

25 mg epothilone A, R_t = 3.5 min (analytical HPLC, 7 µm, column 4 × 250 mm, eluent: see above, flow rate 1.5 mL/min)

and

20 mg 12, 13-bisepi-epothilone A, R_t = 3.7 min, ESI-MS (positive ions)

m/z = 494 $[M + H]^+$

^{1}H-NMR in $[D_4]$ methanol, selected signals:

δ = 4.32 (3-H), 3.79 (7-H), 3.06 (12-H), 3.16 (13-H), 5.54 (15-H), 6.69 (17-H), 1.20 (22-H), 1.45 (23-H).

[insert]

12, 13-bisepi-epothilone A, R = H

Example 3:

Epothilones E and F, new biotransformation products of epothilones A and B.

Production strain:

In July 1985, the production strain *Sorangium cellulosum* So ce90 was isolated at GBF from a soil sample obtained from beaches in Zambesi; this strain was then deposited on October 28, 1991 with the German Collection for Microorganisms under the number DSM 6773.

The following sources describe the characterization of the producing cells and the culture conditions:

G. Höfle, N. Bedorf, K. Gerth and H. Reichenbach: Epothilones, their synthesis methods and agents containing them. German Patent Application 41 38 042 A1, laid open for public inspection on May 27, 1993.

Formation of epothilones E and E [sic; F] during fermentation:

A typical fermentation proceeds as follows: A 100 L bioreactor is charged with 60 L medium (0.8% starch, 0.2% glucose, 0.2% soybean meal, 0.2% yeast extract, 0.1% $CaCl_2$ x $2H_2O$, 0.1% $MgSO_4$ x $7H_2O$, 8 mg/L Fe-EDTA, pH 7.4). In addition, 2% adsorber resin (XAD-16, Rohm & Haas) is added. The medium is sterilized by autoclaving (2 hours, 120°C), then inoculated with 10 L of a preculture grown in an agitator flask (160 rpm, 30°C) in the same medium (plus 50 mM HEPES buffer, pH 7.4). Fermentation is performed at 32°C at an agitator speed of 500 rpm and with an aeration rate of 0.2 L [STP] per m^3 and per hour, and the pH is kept at 7.4 by adding KOH. Fermentation lasts for 7 to 10 days. The epothilones thus formed are bound continuously to the adsorber resin during fermentation. After separating the culture medium (e.g., by screening in a process filter), the resin is washed with three bed volumes of water and eluted with four bed volumes of methanol. The eluate is concentrated until dry and dissolved in 700 mL methanol.

HPLC analysis of the XAD eluate:

The eluate is concentrated 100:1 in relation to the starting volume of the reactor (70 L). The analysis is performed with an HPLC system 1090 from Hewlett Packard. A Microbore column (125/2 Nucleosil 120-5 C_{18}) from Machery-Nagel (Düren) is used to separate the components. Elution is performed with a water/acetonitrile gradient from 75:25 initially to 50:50 after 5.5 minutes. This ratio is maintained until the 7^{th} minute and then is increased to 100% acetonitrile by the 10^{th} minute.

The measurement is performed at a wavelength of 250 nm and a bandwidth of 4 nm. The diode array spectra are measured in the wavelength range of 200 to 400 nm. In the XAD eluate, two new substances with R_t = 5.29 and R_t = 5.91 occur, their absorption spectra being identical to those of epothilones A and/or B (Figure 1; E corresponds to A; F corresponds to B). Under the given fermentation conditions, these substances are formed only in traces.

Biotransformation of epothilones A and B to epothilones E and F:

A four-day-old 500 mL culture of So ce90 kept with adsorber resin is used for the controlled biotransformation. A 250 mL portion of this is transferred to a sterile 1 L Erlenmeyer flask,

leaving the XAD. Then a methanolic solution of a mixture of a total of 36 mg epothilone A and 14 mg epothilone B is added and the flask is incubated for 2 days at 30°C and 200 rpm on an agitator. The formation of epothilones E and F is analyzed directly from 10 µL of the centrifuged culture supernatant (Figure 2). The transformation occurs only in the presence of the cells and is a function of the cell density and time. Figure 3 shows the kinetics of this transformation in the case of epothilone A.

Isolation of epothilones E and F

For isolation of epothilones E and F, three agitated flask batches from biotransformation (see above) are combined and agitated for one hour with 20 mL XAD-16. The XAD is recovered by screening and is eluted with 200 mL methanol. The eluate is evaporated *in vacuo* to yield 1.7 g crude extract, which is distributed between 30 mL ethyl acetate and 100 mL water. By evaporating *in vacuo*, 330 mg of an oily residue is obtained from the ethyl acetate phase and chromatographed in five runs over a 250 x 20 mm RP-18 column (eluent: methanol/water 58:42, detection 254 nm).

Yield: Epothilone E 50 mg
Epothilone F 10 mg

Biological effect of epothilone E:

In cell cultures with a concentration that yields a 50% reduction in growth was determined (IC_{50}) and compared with the values for epothilone A.

Cell line	IC_{50} (ng/mL)	
	Epothilone E	Epothilone A
HeLa KB-3.1 (human)	5	1
Mouse fibroblasts, L929	20	4

Epothilone E

$C_{26}H_{39}HO_7S$ [509]
ESI-MS (positive ions): 510.3 for $[M + H]^+$

TLC: $R_f = 0.58$
TLC aluminum foil 60 F 254 Merck. Eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.

HPLC: R_t = 5.0 min
Column: Nucleosil 100 C-18 7µm, 250 × 4 mm
Eluent: methanol/water = 60:40
Flow rate: 1.2 mL/min
Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

Epothilone F

$C_{27}H_{41}NO_7S$ [523]
ESI-MS (positive ions): 524.5 for $[M + H]^+$

TLC: R_f = 0.58
TLC aluminum foil 60 F 254 Merck. Eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.
HPLC: R_t = 5.4 min
Column: Nucleosil 100 C-18 7µm, 250 × 4 mm
Eluent: methanol/water = 60:40
Flow rate: 1.2 mL/min
Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Example 4:

Producing epothilones E and F by biotransformation with *Sorangium cellulosum* So ce90

1) Performing the biotransformation:

A culture of *Sorangium cellulosum* So ce90, which was agitated for 4 days at 160 rpm and 30°C in the presence of 2% XAD-16 adsorber resin (Rohm & Haas, Frankfurt/Main), was used for the biotransformation. The culture medium had the following composition in g/L distilled water: potato starch (Maizena), 8; glucose (Maizena), 8; defatted soybean meal, 2; yeast extract (Marcor), 2; ethylenediamine tetraacetic acid, iron (III) sodium salt, 0.008; $MgSO_4 \times 7H_2O$, 1; $CaCl_2 \times 2H_2O$, 1; HEPES 11.5. The pH was adjusted with KOH to 7.4 before autoclaving. XAD was removed from the culture by screening through a stainless steel screen (200 µM mesh). The bacteria were sedimented by centrifugation for 10 minutes at 10,000 rpm, and the pellet was resuspended in 1/5 of the culture supernatant. Then epothilone A or epothilone B in methanolic solution in a concentration of 0.5 g/L was added to the concentrated bacterial suspension. Culturing was continued as described above. For analysis of the biotransformation, a 1 mL sample was taken at the desired times, 0.1 mL XAD was added, and the sample was agitated for 30 min at 30°C. The XAD was eluted with methanol. The eluate was concentrated until dry and redissolved in 0.2 mL methanol. The sample was analyzed by HPLC.

Figure 4. Kinetics of a biotransformation of epothilone A to epothilone E.

Figure 5. Kinetics of a biotransformation of epothilone B to epothilone F.

2) Production of epothilone E by biotransformation of 1 g epothilone A

The *Sorangium cellulosum* So ce90 strain was cultured for 4 days in 8.5 L of the above medium (but without the addition of XAD) in a 10 L bioreactor at 30°C, a rotational speed of 150 rpm and with an aeration rate of 0.1 vvm.

Then the culture was concentrated to 3 L by cross-flow filtration using a membrane (0.6 m^2) having a pore size of 0.3 µm.

The concentrated culture was transferred to a 4 L bioreactor, and a methanolic solution of 1 g epothilone A in 10 mL methanol was added. Culturing was then continued for a period of 21.5 hours. The temperature was 32°C, the agitator speed was 455 rpm and the aeration rate was 6 liters per minute. At the time of harvest, 100 mL XAD was added and incubation was continued for one hour. XAD was separated by screening and eluted exhaustively with methanol. The concentrated eluate was analyzed by HPLC.

Balance calculations for the biotransformation:

Epothilone A used:	1000 mg	=	100%
Epothilone A recovered after 21.5 hours:	53.7 mg	=	5.4%
Epothilone E formed after 21.5 hours:	661.4 mg	=	66.1%
Epothilone A completely degraded:		=	28.5%

Experiment 5:

The epothilones according to this invention were tested with cell cultures (Table 2) and were tested for promoting polymerization (Table 3).

Table 2:

Epothilone tests with cell cultures.

Epothilone	A 493	B 507	C 477	D 491	E 509	F 523
			IC-50 (ng/mL)			
Mouse fibroblasts L929	4	1	100	20	20	1.5
Human tumor cell lines:						
HL-60 (leukemia)	0.2	0.2	10	3	1	0.3
K-562 (leukemia)	0.3	0.3	20	10	2	0.5
U-937 (lymphoma)	0.2	0.2	10	3	1	0.2
KB-3.1 (cervical carcinoma)	1	0.6	20	12	5	0.5
KB-V1 (cervical carcinoma, multires)	0.3	0.3	15	3	5	0.6
A-498 (renal carcinoma)	–	1.5	150	20	20	3
A-549 (pulmonary carcinoma)	0.7	0.1	30	10	3	0.1

Table 3:

Polymerization test with epothilones.

Parameter: Time until half maximum polymerization of the controls

Measurement:	w	x	y	z	Average (s)	Average (%)
Control	200	170	180	210	190	100
Epothilone A	95	60	70	70	74	39
Epothilone B		23	25	30	26	14
Epothilone C	125	76	95	80	94	49
Epothilone D	125	73	120		106	56
Epothilone E	80	60	50	45	59	31
Epothilone F	80	40	30	50	50	26

Standard test with 0.9 mg tubulin/mL and 1 μM sample concentration

The polymerization test is an *in vitro* test with purified tubulin from swine brain. The analysis is performed photometrically. Polymerization promoting substances such as the epothilones shorten the period until half the maximum polymerization has occurred, i.e., the shorter the time, the more effective the compound; w, x, y and z are four independent experiments. The relative efficacy is expressed in a percentage of the controls in the last column; again the lowest values indicate the best efficacy. This ranking corresponds rather accurately to that found in cell cultures.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Society for
Biotechnological Research
Mascheroder Weg 1
3300 Braunschweig

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM
Name:	Society for Biotechnological Research	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773
Address:	Mascheroder Weg 1 3300 Braunschweig	Date of the deposit or of the transfer[1]: October 28, 1991

III. VIABILITY STATEMENT
The viability of the microorganism identified under II above was tested on October 28, 1991.[2] On that date, the said microorganism was (x)[3] viable ( )[3] no longer viable
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

IV. INTERNATIONAL DEPOSITARY AUTHORITY		
Name:	DSM GERMAN COLLECTION OF MICROORGANISMS AND CELL CULTURES	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): [signature]
Address:	Mascheroder Weg 1 B D-3300 Braunschweig	Date: November 5, 1991

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSM-BP/9 (sole page) 0787

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Society for
Biotechnological Research
Mascheroder Weg 1
3300 Braunschweig

RECEIPT in the case of an original deposit
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR So ce 90	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773
II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: ( ) a scientific description (×) a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts this microorganism identified under I. above, which was received by it on October 28, 1991 (date of original deposit)[1]	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I. above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSM German Collection of Microorganisms and Cell Cultures Address: Mascheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): [signature] Date: November 5, 1991

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Patent Claims

1. Epothilones obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner,
(b) separating the adsorber resin from the culture and washing with a water/methanol mixture,
(c) eluting the washed adsorber resin with methanol and concentrating the eluate to yield a crude extract,
(d) extracting the concentrate thus obtained with ethyl acetate, concentrating the extract and distributing it between methanol and hexane,
(e) concentrating the methanolic phase to yield a raffinate and fractionating the concentrate on a Sephadex column,
(f) obtaining a fraction having metabolic products of the microorganism used,
(g) chromatographing the fraction thus obtained on a C18 reverse phase with a methanol/water mixture and, in chronological order,
- after a first fraction having epothilone A and
- after a second fraction having epothione [sic; epothilone] B,
- obtaining a third fraction having a first additional epothilone, and
- obtaining a fourth fraction having a second additional epothilone, and
(h1) isolating the epothilone of the first additional fraction and/or
(h2) isolating the epothilone of the second additional fraction.

2. Epothilone of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

3. Epothilone C of the formula:

[insert]

Epothilone C, R = H.

4. Epothilone of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

5. Epothilone D of the formula:

[insert]

Epothilone D, R = CH_3

6. Biotransformant of epothilone A, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone A,

(b) incubating the culture mixed with epothilone A and then combining it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting it with methanol and concentrating the eluate to yield a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to an oil,

(e) chromatographing the oil on an reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60:40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 20 min and isolating the biotransformant.

7. Biotransformant of epothilone A according to Claim 6, obtainable by separating a culture three or four or more days old in step (a).

8. Biotransformant of epothilone A according to Claim 6 or 7, obtainable by incubating the culture for one or two or more days in step (b).

9. Compound having the empirical formula $C_{26}H_{39}NO_7S$, characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a),

2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

10. Compound (epothilone E) having the formula:

[insert]

Epothilone E, R = H.

11. Biotransformant of epothilone B, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone B,

(b) incubating the culture mixed with epothilone B and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting it with methanol and concentrating the eluate to yield a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60:40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 24.5 min and isolating the biotransformant.

12. Biotransformant according to Claim 11, obtainable by separating a culture that is three or four or more days old in step (a).

13. Biotransformant according to Claim 11 or 12, obtainable by incubating the culture for one or two or more days in step (b).

14. Compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

15. Compound (epothilone F) of the formula:

[insert]

Epothilone F, R = CH_3.

16. Phytosanitary agent for use in agriculture and forestry and/or gardening, consisting of one or more of the compounds according to one or more of the preceding claims or one or more of these compounds in addition to one or more conventional vehicle(s) and/or diluent(s).

17. Therapeutic agent, in particular for use as a cytostatic agent, consisting of one or more of the compounds according to one or more of the preceding claims or one or more of the compounds according to one or more of the preceding claims plus one or more conventional vehicle(s) and/or diluent(s).

Fig. 1 - 5

Norm. = Standardized
Epothilon = Epothilone
Ersatzblatt (Regel 26) = Replacement page (Rule 26)

Fig. 4 + 5

Gesamtepothilon = Total epothilone

PCT WORLD ORGANIZATION FOR INTELLECTUAL PROPERTY
International Office [SEAL]

INTERNATIONAL PATENT APPLICATION
PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification[6]:
C07D 417/06, 493/04, C12P 17/08, A01N 43/78, A61K 31/425 // (C07D 493/04, 313:00, 303:00)

A1

(11) International Publication Number:
WO 98/22461

(43) International Publication Date:
May 28, 1998 (5/28/98)

(21) International Application No.: PCT/EP97/06442

(22) International Filing Date: November 18, 1997 (11/18/97)

(30) Priority Dates:
196 47 580.5 Nov. 18, 1996 (11/18/96) Germany
197 07 506.1 Feb. 25, 1997 (02/25/97) Germany

(71) Applicant (*for all contracting nations except USA*): GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH (GBF) [COMPANY FOR BIOTECHNOLOGICAL RESEARCH] [Germany/ Germany], Mascheroder Weg 1, D-38124 Braunschweig (Germany).

(72) Inventor; and

(75) Inventor/applicant (*only for USA*): Hans REICHENBACH [Germany/Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany); Gerhard HOFLE [Germany/ Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany). Klaus GERTH [Germany/Germany]; Mascheroder Weg 1, D-38124 Braunschweig (Germany). Heinrich STEINMETZ [Germany/Germany] Mascheroder Weg 1, D-38124 Braunschweig (Germany).

(74) Attorney: Hans D. BOETERS et al.; Boeters & Bauer, Bereiteranger 15, D-81541 Munich (Germany).

(81) Contracting nations: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN; ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW); Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM); European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE); OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With the International Search Report.
If there are any changes before the final date of the period allowed for changes, publication will be repeated.

(54) Title: EPOTHILONES C, D, E AND F, PRODUCTION PROCESS AND THEIR USE AS CYTOSTATIC AS WELL AS PHYTOSANITARY AGENTS

[Insert (57) Abstract in English] [Insert I, II]

FOR INFORMATIONAL PURPOSES ONLY

Codes used to define PCT countries added to page 1 of the pamphlet of unexamined international applications according to the PCT.

AL	Albania	**ES**	Spain	**LT**	Lithuania	**SN**	Senegal
AM	Armenia	**FI**	Finland	**LU**	Luxembourg	**SZ**	Swaziland
AT	Austria	**FR**	France	**LV**	Latvia	**TD**	Chad
AU	Australia	**GA**	Gabon	**MC**	Monaco	**TG**	Togo
AZ	Azerbaijan	**GB**	Great Britain	**MD**	Moldova	**TJ**	Tajikistan
BA	Bosnia-Herzegovina	**GE**	Georgia	**MG**	Madagascar	**TM**	Turkmenistan
BB	Barbados	**GH**	Ghana	**MK**	Macedonia, former Yugoslavia Republic	**TR**	Turkey
BE	Belgium	**GN**	Guinea	**ML**	Mali	**TT**	Trinidad & Tobago
BF	Burkina Paso	**GR**	Greece	**MN**	Mongolia	**UA**	Ukraine
BG	Bulgaria	**HU**	Hungary	**MR**	Mauritania	**UG**	Uganda
BJ	Benin	**IE**	Ireland	**MW**	Republic of Malawi	**US**	United States of America
BR	Brazil	**IL**	Israel	**MX**	Mexico	**UZ**	Republic of Uzbekistan
BY	Belarus	**IS**	Iceland	**NE**	Niger	**VN**	Vietnam
CA	Canada	**IT**	Italy	**NL**	Netherlands	**YU**	Yugoslavia
CF	Central African Republic	**JP**	Japan	**NO**	Norway	**ZW**	Zimbabwe
CG	People's Republic of Congo	**KE**	Kenya	**NZ**	New Zealand		
CH	Switzerland	**KG**	Kyrgyzstan	**PL**	Poland		
CI	Ivory Coast	**KP**	Democratic People's Republic of Korea	**PT**	Portugal		
CM	Cameroon	**KR**	Republic of Korea	**RO**	Romania		
CN	China	**KZ**	Kazakhstan	**RU**	Russian Federation		
CU	Cuba	**LC**	Saint Lucia	**SD**	Sudan		
CZ	Czech Republic	**LI**	Liechtenstein	**SE**	Sweden		
DE	Germany	**LK**	Sri Lanka	**SG**	Singapore		
DK	Denmark	**LR**	Liberia	**SI**	Slovenia		
EE	Estonia	**LS**	Lesotho	**SK**	Slovak Republic		

EPOTHILONES C, D, E AND F, PRODUCTION PROCESS AND THEIR USE AS CYTOSTATIC AS WELL AS PHYTOSANITARY AGENTS

The invention relates to epothilones C, D, E and F, production and use thereof for production of therapeutic agents and phytosanitary agents.

Epothilones C and D

According to one embodiment, this invention relates to epothilones [C and D], which can be produced by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin by an essentially known method,
(b) separating the adsorber resin from the culture and washing it with a water/methanol mixture,
(c) eluting the washed adsorber resin with methanol and concentrating the eluate to yield a crude extract,
(d) extracting the concentrate thus obtained with ethyl acetate, concentrating the extract and distributing it between methanol and hexane,
(e) concentrating the methanolic phase to yield a raffinate and fractionating the concentrate on a Sephadex column,
(f) obtaining a fraction having metabolic products of the microorganism used,
(g) chromatographing the fraction thus obtained with a methanol/water mixture on a C18 reverse phase and, in chronological order,
- after a first fraction having epothilone A and
- after a second fraction having epothione [sic; epothilone] B,
- obtaining a third fraction having a first additional epothilone, and
- obtaining a fourth fraction having a second additional epothilone, and

(h1) isolating the epothilone of the first additional fraction and/or
(h2) isolating the epothilone of the second additional fraction.

Furthermore, this invention relates to an epothilone [C] of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

This invention also relates to epothilone C of the formula:

[insert]

Epothilone C, R = H

Furthermore, this invention relates to epothilone [D] of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

Furthermore, this invention also relates to epothilone D of the formula:

[insert]

Epothilone D, R = CH_3

Epothilones C and D can be used to produce the compounds of the following formula 1, and for their derivatization, reference can be made to the derivatization methods described in WO-A 97/19 086.

[insert]

In formula 1 above, the following meanings are used:

R = H, C_{1-4} alkyl;
R^1, R^2, R^3, R^4, R^5 = H, C_{1-6} alkyl,
C_{1-6} acyl-benzoyl,
C_{1-4} trialkylsilyl,
benzyl,
phenyl,
C_{1-6} alkoxy,
C_6 alkyl-, hydroxy- and halogen-
substituted benzyl or phenyl;

where two of the R^1 through R^5 groups may also be combined to form the $-(CH_2)_n-$ group, where n = 1 to 6, and the alkyl and/or acyl groups contained in the groups may be linear or branched; Y and Z are either the same or different, each standing for hydrogen, halogen, e.g., F, Cl, Br or I, pseudohalogen, e.g., -NCO, -NCS or $-N_3$, OH, O-(C_{1-6})-acyl, O-(C_{1-6})-alkyl, O-benzyl. Y and Z may also denote the O atom of an epoxy, in which case epothilone A and B are not claimed here, or they may form one of the C-C bonds of a C=C double bond.

Thus the 12, 13-double bond may be selectively:
- hydrogenated, e.g., catalytically or with diimine, yielding a compound of formula 1 where Y = Z = H; or
- epoxidized, e.g., with dimethyldioxirane or a peracid, yielding a compound of formula 1 where Y and Z = -O-; or
- converted to the dihalides, dipseudohalides or diazides, yielding a compound of formula 1, where Y and Z = Hal, pseudo-Hal or N_3.

Epothilones E and F

According to another embodiment, this invention relates to a biotransformant of epothilone A obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone A,

(b) incubating the culture mixed with epothilone A and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting with methanol and concentrating the eluate to form a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to yield an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 20 min and isolating the biotransformant.

Furthermore, this invention relates to such a biotransformant as epothilone A, which is obtainable by separating at step (a) a culture, which is three or four or more days old.

Furthermore, this invention relates to such a biotransformant of epothilone A, which is obtainable by incubating the culture for one or two or more days in step (b).

Furthermore, this invention relates to a compound of the empirical formula $C_{26}H_{39}NO_7S$ characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

Furthermore, this invention relates to a compound (epothilone E) of the formula:

[insert]

Epothilone E, R = H

According to another embodiment, this invention relates to a biotransformant of epothilone B, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone B,

(b) incubating the culture mixed with epothilone B and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting with methanol and concentrating the eluate to form a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to yield an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7 µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60 : 40
flow rate:	10 mL/min

and separating the fractions which contain biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 24.5 min and isolating the biotransformant.

Furthermore, this invention relates to such a biotransformant as epothilone B, which is obtainable by incubating a culture which is three or four or more days old in step (a).

Furthermore, this invention relates to such a biotransformant of epothilone B, which is obtainable by incubating the culture for one or two or more days in step (b).

Furthermore, this invention relates to a compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^{1}H-NMR spectral data (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Furthermore, this invention relates to a compound (epothilone F) of the formula:

[insert]

Epothilone F, R = CH_3

Synthesis and agents

The compounds according to this invention, i.e., epothilones, are obtainable by the measures described above.

This invention also relates to phytosanitary agents for use in agriculture, forestry and/or gardening, consisting of one or more of the epothilones C, D, E and F listed above and/or consisting of one or more of the epothilones listed above plus one or more conventional vehicle(s) and/or diluent(s).

Finally, this invention relates to therapeutic agents consisting of one or more of the compounds listed above or one or more of the compounds listed above plus one or more conventional vehicle(s) and/or diluent(s). These agents may have cytotoxic activities in particular and/or may induce immunosuppression and/or be used to combat malignant tumors, but they can be used especially preferably as cytostatics.

This invention is explained in greater detail below through the description of a few selected exemplary embodiments.

Examples

Example 1:
Epothilones C and D

A. Production strain and culture conditions according to the epothilone basic patent, German Patent 41 38 042 B.

B. Production with DSM 6773

75 liters of culture are cultured as described in the basic patent and used in production fermenter to inoculate 700 L of production medium consisting of 0.8% starch, 0.2% glucose, 0.2% soybean meal, 0.2% yeast extract, 0.1% $CaCl_2$ x $2H_2O$, 0.1% $MgSO_4$ x $7H_2O$, 8 mg/L Fe-EDTA, pH = 7.4 and optionally 15 L of Amberlite XAD-16 adsorber resin. The fermentation lasts for seven to ten days at 30°C, aeration rate 0.1 L [STP]/m^3. The pO_2 is kept at 30% by regulating the rotational speed.

C. Isolation

The adsorber resin is separated from the culture with a 100 mesh process filter (0.7 m^2) and freed of polar impurities by washing with three bed volumes of water/methanol 2:1. By elution with four bed volumes of methanol, a crude extract is obtained and then evaporated *in vacuo* until the aqueous phase appears. This phase is extracted three times with an equal volume of ethyl acetate. Evaporating the organic phase yields 240 g crude extract, which is distributed between methanol and heptane to separate the lipophilic impurities. By evaporating *in vacuo*, 180 g raffinate is obtained from the methanol phase and then is fractionated in three portions over Sephadex LH-20 (column 20 × 100 cm, 20 mL/min methanol). The epothilones are contained in the fraction eluted with a retention time of 240 to 300 minutes, this fraction amounting to a total of 72 g. To separate the epothilones, this fraction is chromatographed in three portions on Lichrosorb RP-18 (15 µm, column 10 × 40 cm, eluent 180 mL/min methanol/water 65:35). After epothilones A and B, epothilone C is eluted with R_t = 90 to 95 min, and epothilone D is eluted with R_t = 100 to 110 min. After evaporating *in vacuo*, each is obtained as a colorless oil in a yield of 0.3 g.

D. Physical properties

[insert]

Epothilone C, R = H
Epothilone D, R = CH_3

Epothilone C

$C_{26}H_{39}NO_5S$ [477]
ESI-MS (positive ions): 478.5 for $[M + H]^+$

^1H and ^{13}C: see NMR table.

TLC: R_f = 0.82
TLC aluminum foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1

Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.

HPLC: R_t = 11.5 min
Column: Nucleosil 100 C-18 7µm, 125 × 4 mm
Eluent: methanol/water = 65:35
Flow rate: 1 mL/min
Detection: diode array

Epothilone D

$C_{27}H_{41}NO_5S$ [491]
ESI-MS (positive ions): 492.5 for $[M + H]^+$

^1H and ^{13}C: see NMR table.

TLC: R_f = 0.82
TLC aluminum foil 60 F 254 Merck, eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.
HPLC: R_t = 15.3 min
Column: Nucleosil 100 C-18 7µm, 125 × 4 mm
Eluent: methanol/water = 65:35
Flow rate: 1 mL/min
Detection: diode array

Table 1. ^{1}H and ^{13}C NMR data on epothilone C and epothilone D in [D_6] DMSO at 300 MHz.

H atom	Epothilone C			Epothilone D		
	δ (ppm)	C atom	δ (ppm)	δ (ppm)	C atom	δ (ppm)
		1	170.3		1	170.1
2-Ha	2.38	2	38.4	2.35	2	39.0
2-Hb	2.50	3	71.2	2.38	3	70.8
3-H	3.97	4	53.1	4.10	4	53.2
3-OH	5.12	5	217.1	5.08	5	217.4
6-H	3.07	6	45.4	3.11	6	44.4
7-H	3.49	7	75.9	3.48	7	75.5
7-OH	4.46	8	35.4	4.46	8	36.3
8-H	1.34	9	27.6	1.29	9	29.9
9-Ha	1.15	10	30.0	1.14	10	25.9
9-Hb	1.40	11	27.6	1.38	11	31.8*
10-Ha	1.15*	12	124.6	1.14*	12	
10-Hb	1.35*	13	133.1	1.35*	13	138.3
11-Ha	1.90	14	31.1	1.75	14	120.3
11-Hb	2.18	15	76.3	2.10	15	31.6*
12-H	5.38**	16	137.3		16	76.6
13-H	5.44**	17	119.1	5.08	17	137.2
14-Ha	2.35	18	152.1	2.30	18	119.2
14-Hb	2.70	19	117.7	2.65	19	152.1
15-H	5.27	20	164.2	5.29	20	117.7
17-H	6.50	21	18.8	6.51	21	164.3
19-H	7.35	22	20.8	7.35	22	18.9
21-H_3	2.65	23	22.6	2.65	23	19.7
22-H_3	0.94	24	16.7	0.90	24	22.5
23-H_3	1.21	25	18.4	1.19	25	16.4
24-H_3	1.06	27	14.2	1.07	26	18.4
25-H_3	0.90			0.91	27	22.9
26-H_3				1.63		14.1
27-H_3	2.10			2.11		

*, ** Assignment interchangeable

Example 2:
Epothilone A and 12, 13-bisepi-epothilone A from epothilone C

50 mg epothilone A is dissolved in 1.5 mL acetone and mixed with 1.5 mL of a 0.07 molar solution of dimethyldioxirane in acetone. After standing for six hours at room temperature, the mixture is evaporated *in vacuo* and separated by preparative HPLC on silica gel (eluent: methyl-tert-butyl ether/petroleum ether/methanol 33:66:1).

Yield:

25 mg epothilone A, R_t = 3.5 min (analytical HPLC, 7 µm, column 4 × 250 mm, eluent: see above, flow rate 1.5 mL/min)

and

20 mg 12, 13-bisepi-epothilone A, R_t = 3.7 min, ESI-MS (positive ions)

m/z = 494 $[M + H]^+$
^{1}H-NMR in $[D_4]$ methanol, selected signals:
δ = 4.32 (3-H), 3.79 (7-H), 3.06 (12-H), 3.16 (13-H), 5.54 (15-H), 6.69 (17-H), 1.20 (22-H), 1.45 (23-H).

[insert]

12, 13-bisepi-epothilone A, R = H

Example 3:
Epothilones E and F, new biotransformation products of epothilones A and B.

Production strain:
In July 1985, the production strain *Sorangium cellulosum* So ce90 was isolated at GBF from a soil sample obtained from beaches in Zambesi; this strain was then deposited on October 28, 1991 with the German Collection for Microorganisms under the number DSM 6773.

The following sources describe the characterization of the producing cells and the culture conditions:

G. Höfle, N. Bedorf, K. Gerth and H. Reichenbach: Epothilones, their synthesis methods and agents containing them. German Patent Application 41 38 042 A1, laid open for public inspection on May 27, 1993.

Formation of epothilones E and E [sic; F] during fermentation:

A typical fermentation proceeds as follows: A 100 L bioreactor is charged with 60 L medium (0.8% starch, 0.2% glucose, 0.2% soybean meal, 0.2% yeast extract, 0.1% $CaCl_2$ x $2H_2O$, 0.1% $MgSO_4$ x $7H_2O$, 8 mg/L Fe-EDTA, pH 7.4). In addition, 2% adsorber resin (XAD-16, Rohm & Haas) is added. The medium is sterilized by autoclaving (2 hours, 120°C), then inoculated with 10 L of a preculture grown in an agitator flask (160 rpm, 30°C) in the same medium (plus 50 mM HEPES buffer, pH 7.4). Fermentation is performed at 32°C at an agitator speed of 500 rpm and with an aeration rate of 0.2 L [STP] per m^3 and per hour, and the pH is kept at 7.4 by adding KOH. Fermentation lasts for 7 to 10 days. The epothilones thus formed are bound continuously to the adsorber resin during fermentation. After separating the culture medium (e.g., by screening in a process filter), the resin is washed with three bed volumes of water and eluted with four bed volumes of methanol. The eluate is concentrated until dry and dissolved in 700 mL methanol.

HPLC analysis of the XAD eluate:

The eluate is concentrated 100:1 in relation to the starting volume of the reactor (70 L). The analysis is performed with an HPLC system 1090 from Hewlett Packard. A Microbore column (125/2 Nucleosil 120-5 C_{18}) from Machery-Nagel (Düren) is used to separate the components. Elution is performed with a water/acetonitrile gradient from 75:25 initially to 50:50 after 5.5 minutes. This ratio is maintained until the 7^{th} minute and then is increased to 100% acetonitrile by the 10^{th} minute.

The measurement is performed at a wavelength of 250 nm and a bandwidth of 4 nm. The diode array spectra are measured in the wavelength range of 200 to 400 nm. In the XAD eluate, two new substances with R_t = 5.29 and R_t = 5.91 occur, their absorption spectra being identical to those of epothilones A and/or B (Figure 1; E corresponds to A; F corresponds to B). Under the given fermentation conditions, these substances are formed only in traces.

Biotransformation of epothilones A and B to epothilones E and F:

A four-day-old 500 mL culture of So ce90 kept with adsorber resin is used for the controlled biotransformation. A 250 mL portion of this is transferred to a sterile 1 L Erlenmeyer flask,

leaving the XAD. Then a methanolic solution of a mixture of a total of 36 mg epothilone A and 14 mg epothilone B is added and the flask is incubated for 2 days at 30°C and 200 rpm on an agitator. The formation of epothilones E and F is analyzed directly from 10 µL of the centrifuged culture supernatant (Figure 2). The transformation occurs only in the presence of the cells and is a function of the cell density and time. Figure 3 shows the kinetics of this transformation in the case of epothilone A.

Isolation of epothilones E and F

For isolation of epothilones E and F, three agitated flask batches from biotransformation (see above) are combined and agitated for one hour with 20 mL XAD-16. The XAD is recovered by screening and is eluted with 200 mL methanol. The eluate is evaporated *in vacuo* to yield 1.7 g crude extract, which is distributed between 30 mL ethyl acetate and 100 mL water. By evaporating *in vacuo*, 330 mg of an oily residue is obtained from the ethyl acetate phase and chromatographed in five runs over a 250 × 20 mm RP-18 column (eluent: methanol/water 58:42, detection 254 nm).

Yield: Epothilone E 50 mg
Epothilone F 10 mg

Biological effect of epothilone E:

In cell cultures with a concentration that yields a 50% reduction in growth was determined (IC_{50}) and compared with the values for epothilone A.

Cell line	IC_{50} (ng/mL)	
	Epothilone E	Epothilone A
HeLa KB-3.1 (human)	5	1
Mouse fibroblasts, L929	20	4

Epothilone E

$C_{26}H_{39}HO_7S$ [509]
ESI-MS (positive ions): 510.3 for $[M + H]^+$

TLC: $R_f = 0.58$
TLC aluminum foil 60 F 254 Merck. Eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.

HPLC: R_t = 5.0 min
Column: Nucleosil 100 C-18 7µm, 250 × 4 mm
Eluent: methanol/water = 60:40
Flow rate: 1.2 mL/min
Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a), 2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

Epothilone F

$C_{27}H_{41}NO_7S$ [523]
ESI-MS (positive ions): 524.5 for $[M + H]^+$

TLC: R_f = 0.58
TLC aluminum foil 60 F 254 Merck. Eluent: dichloromethane/methanol = 9:1
Detection: UV extinction at 254 nm. Spraying with vanillin-sulfuric acid reagent, blue-gray color on heating to 120°C.
HPLC: R_t = 5.4 min
Column: Nucleosil 100 C-18 7µm, 250 × 4 mm
Eluent: methanol/water = 60:40
Flow rate: 1.2 mL/min
Detection: diode array

^{1}H-NMR (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

Example 4:

Producing epothilones E and F by biotransformation with *Sorangium cellulosum* So ce90

1) Performing the biotransformation:

A culture of *Sorangium cellulosum* So ce90, which was agitated for 4 days at 160 rpm and 30°C in the presence of 2% XAD-16 adsorber resin (Rohm & Haas, Frankfurt/Main), was used for the biotransformation. The culture medium had the following composition in g/L distilled water: potato starch (Maizena), 8; glucose (Maizena), 8; defatted soybean meal, 2; yeast extract (Marcor), 2; ethylenediamine tetraacetic acid, iron (III) sodium salt, 0.008; $MgSO_4 \times 7H_2O$, 1; $CaCl_2 \times 2H_2O$, 1; HEPES 11.5. The pH was adjusted with KOH to 7.4 before autoclaving. XAD was removed from the culture by screening through a stainless steel screen (200 µM mesh). The bacteria were sedimented by centrifugation for 10 minutes at 10,000 rpm, and the pellet was resuspended in 1/5 of the culture supernatant. Then epothilone A or epothilone B in methanolic solution in a concentration of 0.5 g/L was added to the concentrated bacterial suspension. Culturing was continued as described above. For analysis of the biotransformation, a 1 mL sample was taken at the desired times, 0.1 mL XAD was added, and the sample was agitated for 30 min at 30°C. The XAD was eluted with methanol. The eluate was concentrated until dry and redissolved in 0.2 mL methanol. The sample was analyzed by HPLC.

Figure 4. Kinetics of a biotransformation of epothilone A to epothilone E.

Figure 5. Kinetics of a biotransformation of epothilone B to epothilone F.

2) Production of epothilone E by biotransformation of 1 g epothilone A

The *Sorangium cellulosum* So ce90 strain was cultured for 4 days in 8.5 L of the above medium (but without the addition of XAD) in a 10 L bioreactor at 30°C, a rotational speed of 150 rpm and with an aeration rate of 0.1 vvm.

Then the culture was concentrated to 3 L by cross-flow filtration using a membrane (0.6 m^2) having a pore size of 0.3 µm.

The concentrated culture was transferred to a 4 L bioreactor, and a methanolic solution of 1 g epothilone A in 10 mL methanol was added. Culturing was then continued for a period of 21.5 hours. The temperature was 32°C, the agitator speed was 455 rpm and the aeration rate was 6 liters per minute. At the time of harvest, 100 mL XAD was added and incubation was continued for one hour. XAD was separated by screening and eluted exhaustively with methanol. The concentrated eluate was analyzed by HPLC.

Balance calculations for the biotransformation:

Epothilone A used:	1000 mg	=	100%
Epothilone A recovered after 21.5 hours:	53.7 mg	=	5.4%
Epothilone E formed after 21.5 hours:	661.4 mg	=	66.1%
Epothilone A completely degraded:		=	28.5%

Experiment 5:

The epothilones according to this invention were tested with cell cultures (Table 2) and were tested for promoting polymerization (Table 3).

Table 2:

Epothilone tests with cell cultures.

Epothilone	A 493	B 507	C 477	D 491	E 509	F 523
			IC-50 (ng/mL)			
Mouse fibroblasts L929	4	1	100	20	20	1.5
Human tumor cell lines:						
HL-60 (leukemia)	0.2	0.2	10	3	1	0.3
K-562 (leukemia)	0.3	0.3	20	10	2	0.5
U-937 (lymphoma)	0.2	0.2	10	3	1	0.2
KB-3.1 (cervical carcinoma)	1	0.6	20	12	5	0.5
KB-V1 (cervical carcinoma, multires)	0.3	0.3	15	3	5	0.6
A-498 (renal carcinoma)	–	1.5	150	20	20	3
A-549 (pulmonary carcinoma)	0.7	0.1	30	10	3	0.1

Table 3:

Polymerization test with epothilones.

Parameter: Time until half maximum polymerization of the controls

Measurement:	w	x	y	z	Average (s)	Average (%)
Control	200	170	180	210	190	100
Epothilone A	95	60	70	70	74	39
Epothilone B		23	25	30	26	14
Epothilone C	125	76	95	80	94	49
Epothilone D	125	73	120		106	56
Epothilone E	80	60	50	45	59	31
Epothilone F	80	40	30	50	50	26

Standard test with 0.9 mg tubulin/mL and 1 µM sample concentration

The polymerization test is an *in vitro* test with purified tubulin from swine brain. The analysis is performed photometrically. Polymerization promoting substances such as the epothilones shorten the period until half the maximum polymerization has occurred, i.e., the shorter the time, the more effective the compound; w, x, y and z are four independent experiments. The relative efficacy is expressed in a percentage of the controls in the last column; again the lowest values indicate the best efficacy. This ranking corresponds rather accurately to that found in cell cultures.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Society for
Biotechnological Research
Mascheroder Weg 1
3300 Braunschweig

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM
Name:	Society for Biotechnological Research	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773
Address:	Mascheroder Weg 1 3300 Braunschweig	Date of the deposit or of the transfer[1]: October 28, 1991

III. VIABILITY STATEMENT
The viability of the microorganism identified under II above was tested on October 28, 1991.[2] On that date, the said microorganism was (x)[3] viable ( )[3] no longer viable
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

IV. INTERNATIONAL DEPOSITARY AUTHORITY		
Name:	DSM GERMAN COLLECTION OF MICROORGANISMS AND CELL CULTURES	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): [signature]
Address:	Mascheroder Weg 1 B D-3300 Braunschweig	Date: November 5, 1991

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2 (a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSM-BP/9 (sole page) 0787

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Society for
Biotechnological Research
Mascheroder Weg 1
3300 Braunschweig

RECEIPT in the case of an original deposit
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR So ce 90	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 6773
II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: ( ) a scientific description (x) a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts this microorganism identified under I. above, which was received by it on October 28, 1991 (date of original deposit)[1]	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I. above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSM German Collection of Microorganisms and Cell Cultures Address: Mascheroder Weg 1 B D-3300 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): [signature] Date: November 5, 1991

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Patent Claims

1. Epothilones obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner,
(b) separating the adsorber resin from the culture and washing with a water/methanol mixture,
(c) eluting the washed adsorber resin with methanol and concentrating the eluate to yield a crude extract,
(d) extracting the concentrate thus obtained with ethyl acetate, concentrating the extract and distributing it between methanol and hexane,
(e) concentrating the methanolic phase to yield a raffinate and fractionating the concentrate on a Sephadex column,
(f) obtaining a fraction having metabolic products of the microorganism used,
(g) chromatographing the fraction thus obtained on a C18 reverse phase with a methanol/water mixture and, in chronological order,
– after a first fraction having epothilone A and
– after a second fraction having epothione [sic; epothilone] B,
– obtaining a third fraction having a first additional epothilone, and
– obtaining a fourth fraction having a second additional epothilone, and
(h1) isolating the epothilone of the first additional fraction and/or
(h2) isolating the epothilone of the second additional fraction.

2. Epothilone of the empirical formula $C_{26}H_{39}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

3. Epothilone C of the formula:

[insert]

Epothilone C, R = H.

4. Epothilone of the empirical formula $C_{27}H_{41}NO_5S$, characterized by the ^1H and ^{13}C-NMR spectral data according to Table 1.

5. Epothilone D of the formula:

[insert]

Epothilone D, R = CH_3

6. Biotransformant of epothilone A, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone A,

(b) incubating the culture mixed with epothilone A and then combining it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting it with methanol and concentrating the eluate to yield a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to an oil,

(e) chromatographing the oil on an reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60:40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 20 min and isolating the biotransformant.

7. Biotransformant of epothilone A according to Claim 6, obtainable by separating a culture three or four or more days old in step (a).

8. Biotransformant of epothilone A according to Claim 6 or 7, obtainable by incubating the culture for one or two or more days in step (b).

9. Compound having the empirical formula $C_{26}H_{39}NO_7S$, characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.38 (2-H_a), 2.51 (2-H_b), 4.17 (3-H), 3.19 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.89 (12-H), 3.00 (13-H), 1.88 (14-H_a),

2.07 (14-H_b), 5.40 (15-H), 6.57 (17-H), 7.08 (19-H), 4.85 (21-H_2), 1.05 (22-H_3), 1.32 (23-H_3), 1.17 (24-H_3), 0.97 (25-H_3), 2.04 (27-H_3).

10. Compound (epothilone E) having the formula:

[insert]

Epothilone E, R = H.

11. Biotransformant of epothilone B, obtainable by:

(a) culturing *Sorangium cellulosum* DSM 6773 in the presence of an adsorber resin in an essentially known manner, separating it from the adsorber resin and optionally mixing all or part of the separated culture with a methanolic solution of epothilone B,

(b) incubating the culture mixed with epothilone B and then mixing it with adsorber resin,

(c) separating the adsorber resin from the culture, eluting it with methanol and concentrating the eluate to yield a crude extract,

(d) distributing the crude extract between ethyl acetate and water, separating the ethyl acetate phase and concentrating it to an oil,

(e) chromatographing the oil on a reverse phase under the following conditions:

column material:	Nucleosil 100 C-18 7µm
column dimensions:	250 × 16 mm
eluent:	methanol/water = 60:40
flow rate:	10 mL/min

and separating the fractions containing biotransformant that can be detected by UV extinction at 254 nm with an R_t value of 24.5 min and isolating the biotransformant.

12. Biotransformant according to Claim 11, obtainable by separating a culture that is three or four or more days old in step (a).

13. Biotransformant according to Claim 11 or 12, obtainable by incubating the culture for one or two or more days in step (b).

14. Compound of the empirical formula $C_{27}H_{41}NO_7S$, characterized by the following ^{1}H-NMR spectrum (300 MHz, $CDCl_3$): δ = 2.37 (2-H_a), 2.52 (2-H_b), 4.20 (3-H), 3.27 (6-H), 3.74 (7-H), 1.30 - 1.70 (8-H, 9-H_2, 10-H_2, 11-H_2), 2.78 (13-H), 1.91 (14-H), 2.06 (14-H_b), 5.42 (15-H), 6.58 (17-H), 7.10 (19-H), 4.89 (21-H_2), 1.05 (22-H_3), 1.26 (23-H_3), 1.14 (24-H_3), 0.98 (25-H_3), 1.35 (26-H_3), 2.06 (27-H_3).

15. Compound (epothilone F) of the formula:

[insert]

Epothilone F, R = CH_3.

16. Phytosanitary agent for use in agriculture and forestry and/or gardening, consisting of one or more of the compounds according to one or more of the preceding claims or one or more of these compounds in addition to one or more conventional vehicle(s) and/or diluent(s).

17. Therapeutic agent, in particular for use as a cytostatic agent, consisting of one or more of the compounds according to one or more of the preceding claims or one or more of the compounds according to one or more of the preceding claims plus one or more conventional vehicle(s) and/or diluent(s).

Fig. 1 - 5

Norm. = Standardized
Epothilon = Epothilone
Ersatzblatt (Regel 26) = Replacement page (Rule 26)

Fig. 4 + 5

Gesamtepothilon = Total epothilone